

# Telomerase Mediates Lymphocyte Proliferation but Not the Atherosclerosis-Suppressive Potential of Regulatory T-Cells

Gavin David Richardson, Andrew Sage, Karim Bennaceur, Nayef Al Zhrany, Jose Coelho-Lima, Emily Dookun, Lilia Draganova, Gabriele Saretzki, David T. Breault, Ziad Mallat, Ioakim Spyridopoulos

**Objective**—Atherosclerosis is an age-related disease characterized by systemic oxidative stress and low-grade inflammation. The role of telomerase and telomere length in atherogenesis remains contentious. Short telomeres of peripheral leukocytes are predictive for coronary artery disease. Conversely, attenuated telomerase has been demonstrated to be protective for atherosclerosis. Hence, a potential causative role of telomerase in atherogenesis is critically debated.

**Approach and Results**—In this study, we used multiple mouse models to investigate the regulation of telomerase under oxidative stress as well as its impact on atherogenesis in vitro and in vivo. Using primary lymphocytes and myeloid cell cultures, we demonstrate that cultivation under hyperoxic conditions induced oxidative stress resulting in chronic activation of CD4<sup>+</sup> cells and significantly reduced CD4<sup>+</sup> T-cell proliferation. The latter was telomerase dependent because oxidative stress had no effect on the proliferation of primary lymphocytes isolated from telomerase knockout mice. In contrast, myeloid cell proliferation was unaffected by oxidative stress nor reliant on telomerase. Telomerase reverse transcriptase deficiency had no effect on regulatory T-cell (T<sub>reg</sub>) numbers in vivo or suppressive function ex vivo. Adoptive transfer of telomerase reverse transcriptase<sup>−/−</sup> T<sub>reg</sub> into Rag2<sup>−/−</sup> ApoE<sup>−/−</sup> (recombination activating gene 2/apolipoprotein E) double knockout mice demonstrated that telomerase function was not required for the ability of T<sub>reg</sub> to protect against atherosclerosis. However, telomere length was critical for T<sub>reg</sub> function.

**Conclusions**—Telomerase contributes to lymphocyte proliferation but plays no major role in T<sub>reg</sub> function, provided that telomere length is not critically short. We suggest that oxidative stress may contribute to atherosclerosis via suppression of telomerase and acceleration of telomere attrition in T<sub>reg</sub>.

**Visual Overview**—An online [visual overview](http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.309940/-/DC1) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2018;38:1283-1296. DOI: 10.1161/ATVBAHA.117.309940.)

**Key Words:** atherosclerosis ■ lymphocytes ■ models, animal ■ oxidative stress ■ telomerase

Atherosclerosis is an age-related systemic disease characterized by systemic oxidative stress and low-grade chronic inflammation, which is driven both by innate and adaptive immune responses. In particular, distinct subpopulations of T-cells have opposing roles in the development of atherosclerotic lesions,<sup>1</sup> and an imbalance between pathogenic and regulatory immunity influences plaque development and disease progression. We and others have shown previously that the majority of T-cells in an atherosclerotic lesion are CD4<sup>+</sup> T-helper type 1 cells that produce interferon- $\gamma$ <sup>2-12</sup> and deficiency in either interferon- $\gamma$  or the transcription factor T-bet (required for T-helper type 1 differentiation) attenuates the progression of atherosclerosis in cholesterol-fed low-density

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lipoprotein receptor null mice (*Ldlr*<sup>−/−</sup>). Conversely, reduction or dysfunction of the regulatory T-cell population (T<sub>reg</sub>) leads to increased atherosclerosis.<sup>5,13,14</sup> Furthermore, adoptive transfer of the T<sub>reg</sub> subpopulation into hypercholesterolemic mice reduces lesion development.<sup>5</sup> Recently, it has also been demonstrated that prolonged hypercholesterolemia impairs T<sub>reg</sub> cells but not effector T-cell accumulation in atherosclerotic lesions and reversal of hypercholesterolemia can prevent loss of lesional T<sub>reg</sub> cells.<sup>12</sup> The ratio of regulatory to effector T-cells is, therefore, critical in determining the outcome of atherosclerosis.

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From the Cardiovascular Research Centre, Institute of Genetic Medicine, International Centre for Life (G.D.R., K.B., N.A.Z., J.C.-L., E.D., L.D., I.S.) and Institute for Cell and Molecular Biosciences, The Ageing Biology Centre, Newcastle University Institute for Ageing, Campus for Ageing and Vitality (G.S.), Newcastle University, Newcastle upon Tyne, United Kingdom; Division of Cardiovascular Medicine, Department of Medicine, University of Cambridge, United Kingdom (A.S., Z.M.); Division of Endocrinology, Boston Children's Hospital, Harvard Medical School, MA (D.T.B.); Harvard Stem Cell Institute, Cambridge, MA (D.T.B.); INSERM U970, Paris Cardiovascular Research Center, France (Z.M.); and Université Paris Descartes, Sorbonne Paris Cité, France (Z.M.).

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Correspondence to Gavin David Richardson, BSc, PhD, Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle upon Tyne, NE1 3BZ, United Kingdom, E-mail [Gavin.Richardson@ncl.ac.uk](mailto:Gavin.Richardson@ncl.ac.uk); or Ioakim Spyridopoulos, MD, FESC, Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle upon Tyne, NE1 3BZ, United Kingdom, E-mail [Ioakim.spyridopoulos@newcastle.ac.uk](mailto:Ioakim.spyridopoulos@newcastle.ac.uk)

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## Nonstandard Abbreviations and Acronyms

<b>CHD</b>	coronary heart disease
<b>GFP</b>	green fluorescent protein
<b>T<sub>reg</sub></b>	regulatory T-cell
<b>TERC</b>	telomerase RNA component
<b>TERT</b>	telomerase reverse transcriptase
<b>WT</b>	wild type

Human T<sub>reg</sub> cells do not arise solely from thymic generation but can also be induced by rapid turnover from the memory T-cell pool.<sup>15</sup> Once generated, they are susceptible to apoptosis and have limited replicative potential, which is directly related to telomere length.<sup>16</sup> Telomeres consist of tandem TTAGGG DNA repeats at the ends of chromosomes as well as the shelterin complex. They function to maintain chromosomal integrity during cell division and protect against chromosomal instability. In cells without telomerase activity, telomeres shorten with every cell division because of the end replication problem,<sup>17</sup> as well as because of increased oxidative stress.<sup>18</sup> Telomere shortening can be compensated or slowed down by concomitant activity of telomerase, a ribonucleoprotein composed of an RNA subunit (telomerase RNA component [TERC]), containing the template for telomere repeat addition, and a telomerase reverse transcriptase (TERT) subunit. Accordingly, late-generation telomerase knockout mice (TERT<sup>-/-</sup> or TERC<sup>-/-</sup>) display an age-related phenotype.<sup>19–21</sup>

Regarding its specific role in T-lymphocytes, telomerase levels control the lifespan of T lymphocytes<sup>22</sup> while telomere dysfunction in late-generation TERC<sup>-/-</sup> mice reduces the number and function of T- and B- lymphocytes.<sup>16,20,23,24</sup> The end replication problem is not the only mechanism that can contribute to telomere shortening resulting eventually in cellular senescence. Telomeres have been shown to be favored targets for DNA damage.<sup>25,26</sup> Because oxidative stress plays a major role in chronic inflammatory diseases, it has been suggested that telomere damage may be involved in their pathophysiology. However, the exact mechanisms driving telomere damage and shortening under conditions of chronic oxidative stress are still not fully understood.

Telomere length in leukocytes has been investigated in a variety of clinical studies involving patients with either existing or developing coronary heart disease (CHD).<sup>27–33</sup> There remains a paucity of data on the role of telomerase and its regulation in atherosclerosis. The increased prevalence of atherosclerosis in older people is associated with a decline in the function of lymphoid progenitors and mature T-cells,<sup>34</sup> and shorter telomeres have been observed in leukocytes of human patients with advanced coronary atherosclerosis.<sup>35</sup> However, it is unclear as to whether accelerated telomere shortening or inherited telomere length are the most important influences in the development of atherosclerosis.<sup>36,37</sup>

It has been previously suggested that telomere shortening or dysfunction in telomerase activity in leukocytes, including T-cells, may be causative in the progression of this disease.<sup>29,31</sup> This hypothesis, however, is in direct contradiction to the recent demonstration of Poch et al<sup>38</sup> who have shown that a mouse model that lacks telomerase activity and displays

significantly shorter telomeres is in fact protected from atherosclerosis,<sup>38</sup> as well as the observations that aged rabbits develop less atheroma under a high-cholesterol diet compared with younger animals.<sup>39</sup> Several human studies have also failed to identify any association between telomere length and atherosclerosis<sup>40</sup> or between telomere length and mortality in subjects over the age of 65 years.<sup>41–43</sup> However, it has been suggested that studies showing no correlation have examined older populations<sup>37</sup> while until the age of 60 years there is a correlation between telomere length and mortality which includes cardiovascular deaths.<sup>44</sup> Adding further complexity to the interpretation of the human studies is the fact that factors, including race/ethnicity and sex, all influence outcome.<sup>37</sup>

In this study, we aimed to address some of the controversy surrounding the role of telomerase activity and telomere length with regards to T-cell activation and proliferation. Moreover, we aimed to address the functional requirement of telomerase for regulatory T-cell-mediated atheroprotection. We report here that oxidative stress suppresses telomerase activity and attenuates proliferation of CD4<sup>+</sup> T-lymphocytes but not CD11b<sup>+</sup> myeloid cell expansion. Lack of *Tert* in cells with sufficiently long telomeres within a population of T<sub>reg</sub> T-lymphocytes is not detrimental to their suppressive function. In contrast, short telomeres diminished T<sub>reg</sub> number and function.

## Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. Details of the major resources and detailed methods can be found in the [online-only Data Supplement](#).

## Animals and Ethics

Animal work was authorized and approved by the Cambridge and Newcastle University Ethics review boards. All animal procedures were performed conforming to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Both male and female mice were used in all studies. TERT knockout, generated by Chiang et al<sup>45</sup> (Jax strain B6.129S-Tert tm1Yjc/J), and TERC knockout, generated by Blasco et al<sup>46</sup> (Jax strain B6.Cg-Terc tm1Rdp/J), animals were purchased from Jackson Laboratory, Maine. Generation and initial phenotypic characterization of the *mTert*-GFP (green fluorescent protein) mice have been published previously.<sup>47–51</sup> The *mTert*-GFP mouse<sup>2</sup> contains a reporter cassette in which the expression of the gene for GFP is under the control of a 4.4-kb fragment of the promoter of murine *Tert*. As such GFP expression in this model represents *mTert* promoter activity as an indicator of TERT transcription. Rag2<sup>-/-</sup> ApoE<sup>-/-</sup> (recombination activating gene 2/apolipoprotein E) double knockout mice and CD28<sup>-/-</sup> mice were originally obtained from Charles River. All mice were held under the UK Home office animal licenses PPL 60/3864 or PO11C464C. Details for each line used to obtain the data for each figure are included in Table I in the [online-only Data Supplement](#).

## Splenocyte and CD4 Cell Isolation, Culture, and Growth Curves

Cells were isolated and cultured as described previously.<sup>47</sup> Assessment of CD4<sup>+</sup> cell purity is demonstrated in Figure I in the [online-only Data Supplement](#). Splenocytes were cultured in a 24-well plate (2×10<sup>6</sup> cells/2 mL per well). MACSbead mouse T-cell, CD3 and CD28 antibody coated, expansion beads (Miltenyi 130-093-627) were added to medium as described.<sup>47</sup> TA-65 activator (TA65) is a telomerase activator purified from *Astragalus membranaceus*<sup>52</sup> and provided by TA-Science Inc (New York, NY). BIBR 1532 (Tocris Bioscience), a

telomerase inhibitor,<sup>53</sup> was dissolved in dimethyl sulfoxide and used as the indicated concentration.

### Dihydroethidium and Mitosox Staining

Dihydroethidium and Mitosox are established methods to measure superoxide levels.<sup>54,55</sup> Cells were labelled with 10- $\mu$ M dihydroethidium (Molecular Probes) as described<sup>56</sup> or 5- $\mu$ M Mitosox Red (Molecular Probes).

### Telomeric Repeat Amplification Protocol Polymerase Chain Reaction ELISA

Telomeric Repeat Amplification Protocol kit (Roche) was performed as per the manufacturer's instructions. TERT<sup>-/-</sup> splenocytes and the immortal fibroblast cell line 3T3 were used as negative and positive controls (Figure VI in the [online-only Data Supplement](#)).

### Detection of T<sub>reg</sub>

After isolation, splenocytes were labeled using the T<sub>reg</sub> Detection Kit (Miltenyi Biotec, Auburn, CA) as per manufacturer's instructions. In our hands,  $\geq 98\%$  of CD4<sup>+</sup> T-cells can be identified as T-cells by CD3<sup>+</sup> staining (Figure V in the [online-only Data Supplement](#)).

### Atherosclerosis Experiments

Rag2<sup>-/-</sup> ApoE<sup>-/-</sup> mice were transplanted with 10<sup>7</sup> splenocytes from CD28<sup>-/-</sup> mice and either PBS or 10<sup>6</sup> CD4<sup>+</sup> CD25<sup>+</sup> regulatory T-cells from either Tert<sup>-/-</sup> mice or wild-type (WT) littermates. Mice were fed an atherogenic Western diet (21% fat, 0.15% cholesterol) for 7 weeks. Atherosclerosis was quantified in the aortic root as described previously.<sup>57</sup>

### Statistical Analysis

After a test for normality, statistical analysis was performed as appropriate and indicated in the legend of each figure. Data are presented as mean $\pm$ SEM or as dot for individual experiments with a line representing the median. A Mann-Whitney *U* test was used to compare groups of 2, and 2-way ANOVA with Bonferroni post hoc analysis was used to compare groups of  $\geq 3$ . Statistical significance was set at  $P < 0.05$ .

## Results

### Increased Oxidative Stress Results in Chronic T-Cell Activation and Reduced Proliferation

Increased oxidative stress and acute inflammation as well as increased T-cell activity are key characteristics of atherosclerosis. We have previously established hyperoxia as a model of chronic mild oxidative stress.<sup>18,58,59</sup> In this study, we used hyperoxic culture conditions to study the effect of chronic oxidative stress on lymphocyte growth kinetics and T-cell activation.

Splenocytes or CD4<sup>+</sup> T-cells were cultured for 10 days at either physiological oxygen levels (physoxia, 3% oxygen) or hyperoxia (40% oxygen). Assessment of cell purity is shown in Figure I in the [online-only Data Supplement](#). When cultured under hyperoxic conditions, both the total splenocyte population and CD4<sup>+</sup> T-cells displayed a significantly higher level of dihydroethidium staining intensity indicating increased mitochondrial superoxide levels (Figure 1A). Similar results for splenocytes cultured in high oxygen were obtained with another superoxide-specific probe Mitosox (Figure II in the [online-only Data Supplement](#)).

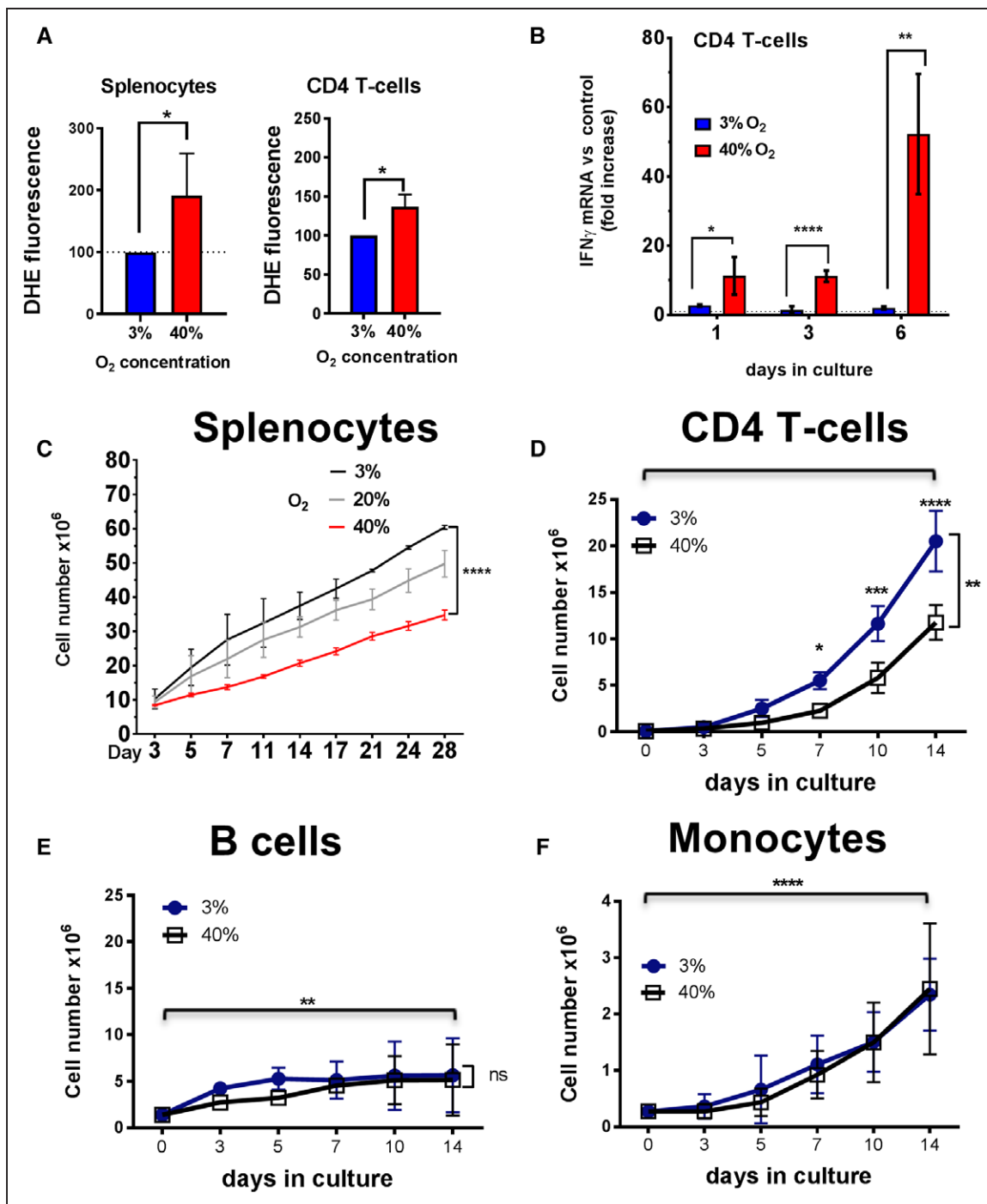
CD4<sup>+</sup> cells cultured under hyperoxic conditions had an elevated interferon  $\gamma$  mRNA expression relative to splenocytes cultured at physoxia (Figure 1B). Increased interferon

$\gamma$  expression was observed throughout the culture duration, indicative of chronic T-cell activation, with a further increase at 6 days. High oxygen had a similar effect on the expression of interleukin 2 (Figure III in the [online-only Data Supplement](#)). Having demonstrated that hyperoxia and oxidative stress are associated with aspects of T-cell activation, we were interested to examine the effect of oxidative stress on splenocyte proliferation. Splenocytes cultured at physoxia demonstrated a 30-fold increase in cell number over 28 days. In contrast, splenocytes cultured in either 20% or 40% oxygen saturation showed a dose-dependent attenuation of proliferation by 15% or 50%, respectively, compared with physoxia ( $P < 0.0001$ ; Figure 1C).

Given the heterogeneity of cell populations residing in the spleen, flow cytometry was used to study the effect of oxidative stress on the proliferation of individual splenocyte subpopulations (Figure 1D–1F). Gating strategy and controls shown in Figure IVA and IVB in the [online-only Data Supplement](#). As CD4 is also expressed on other lymphocyte subpopulations, including dendritic cells, we first quantified what percentage of CD3<sup>+</sup> T-cells is contained within the CD4<sup>+</sup> population. Over 98% of CD4<sup>+</sup> cells also expressed CD3 (Figure V in the [online-only Data Supplement](#)). To reflect the physiological environment, the total splenocyte population was cocultured in the presence of T-cell-specific activation under conditions of physoxia or hyperoxia. After 14 days, total cell numbers of the individual subpopulations were quantified by flow cytometry to extrapolate rates of proliferation. CD4<sup>+</sup> T-cells displayed the greatest proliferative response with 100-fold expansion for 14 days under 3% oxygen (Figure 1D) while a 3-fold increase in B-cell numbers (Figure 1E) and a 7-fold increase in the CD11b<sup>+</sup> population (including myeloid cells, granulocytes, and natural killer T-cells; Figure 1F) was observed during the same period. Importantly, only the proliferation of CD4<sup>+</sup> T-cells was significantly ( $P < 0.01$ ) attenuated by hyperoxia-induced oxidative stress. However, the CD11b<sup>+</sup> were not experimentally stimulated to proliferate under these experimental conditions.

### Oxidative Stress Impairs T-Cell Proliferation via Suppression of Telomerase

We next sought to identify the underlying mechanism by which hyperoxia attenuates T-cell proliferation. We have previously demonstrated that increased oxidative stress excludes the TERT protein from the nucleus of hTERT (human telomerase reverse transcriptase) overexpressing fibroblasts, thereby preventing telomere maintenance and halting cell proliferation<sup>60</sup> and that telomerase facilitates statin-induced T-cell proliferation.<sup>47</sup> The latter led us to hypothesize that the oxidative stress-induced attenuation of T-cell proliferation in this study might be mediated via the suppression of telomerase activity. To test this hypothesis, we quantified telomerase activity in WT splenocytes cultured at either physoxia or hyperoxia. Hyperoxia resulted in a significant reduction of telomerase activity, and all detectable telomerase activity was suppressed after 2 weeks of culture ( $P < 0.0001$ ; Figure 2A). Telomeric Repeat Amplification Protocol assay positive and negative controls are shown in Figure VI in the [online-only Data Supplement](#).

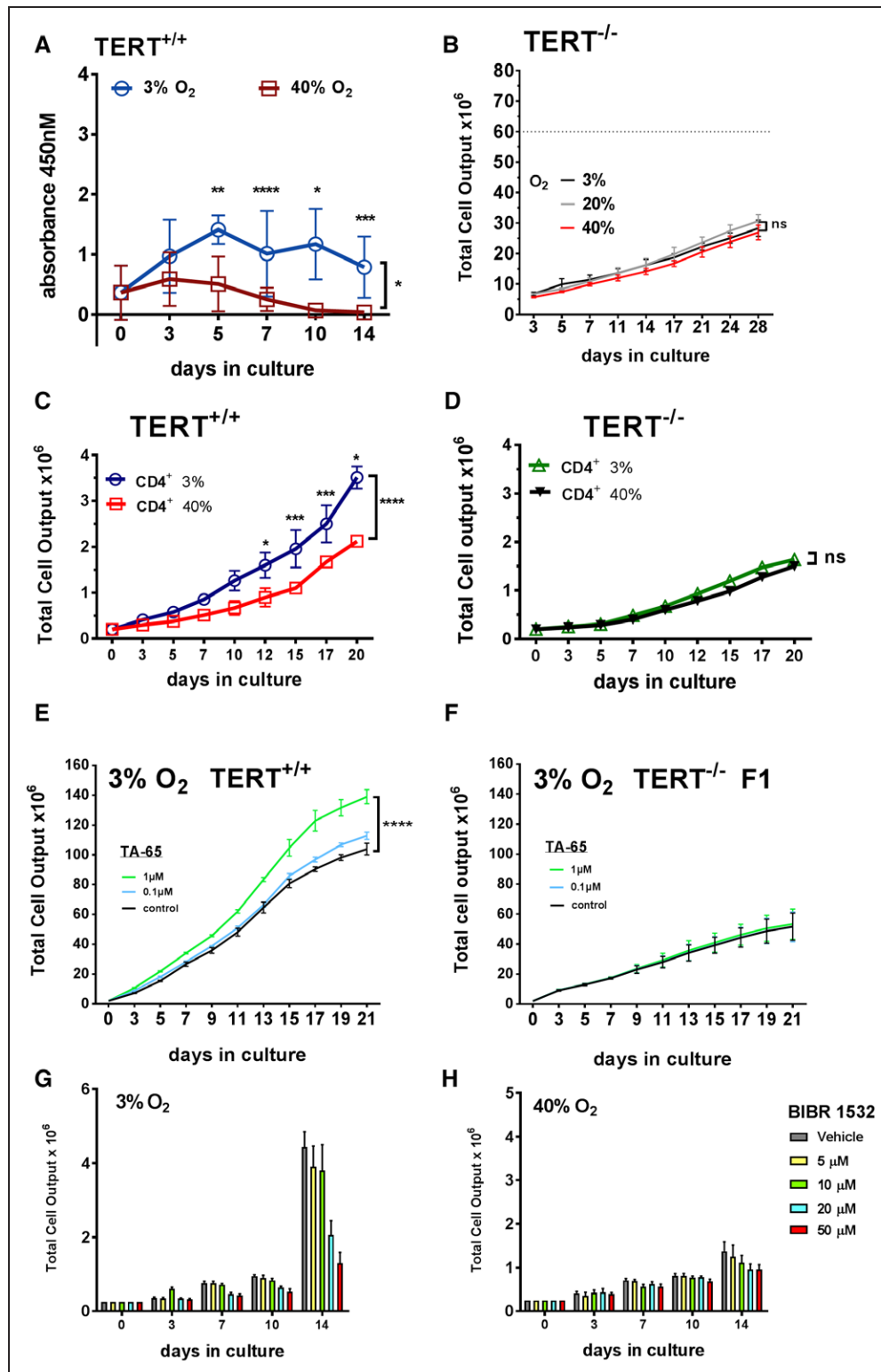


**Figure 1.** Oxidative stress leads to chronic interferon  $\gamma$  (INF- $\gamma$ ) expression and impairs proliferation of CD4 T-cell but not B-cells or myeloid cells. **A**, Total or CD4<sup>+</sup> splenocytes were cultured at either 3% or 40% oxygen saturation for 10 d. Dihydroethidium (DHE) fluorescence was increased under hyperoxia.  $n=4$  for each experimental group. **B**, CD4<sup>+</sup> splenocytes were cultured for up to 6 d at either 3% or 40% oxygen saturation. IFN- $\gamma$  transcript expression was significantly increased at all time points under hyperoxia as quantified by quantitative reverse transcription polymerase chain reaction.  $n=4$  for each experimental group. **C**,  $2 \times 10^5$  splenocytes were cultured per well at 3% or 40% oxygen saturation. Total cell numbers were quantified at 3-d intervals. All error bars represent the standard deviation (SD).  $n=4$  for each experimental group. **D** and **F**, Flow cytometry was used to identify and quantify individual cellular subpopulations of the splenocyte cell cultures. A total of  $2 \times 10^6$  splenocytes/mL were cultured after T-cell-specific activation. Total numbers of each subpopulation were quantified by flow cytometry.  $n=3$  for each experimental group, error bars (SD). \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , and \*\*\*\* $P<0.0001$  using with Mann-Whitney  $U$  test or 2-way ANOVA as appropriate.

To specifically address the role of telomerase in mediating proliferation, we investigated the proliferative potential of splenocytes isolated from first generation (F1) TERT knockout mice, which lack expression of telomerase reverse

transcriptase (Tert<sup>-/-</sup>), the catalytic subunit of telomerase.<sup>61</sup> Although these mice lack functional telomerase activity, they do not have significantly shorter telomeres than WT animals.<sup>47</sup> No significant difference in the rates of proliferation in





**Figure 2.** Oxidative stress decreases T-cell proliferation and suppresses telomerase. **A**,  $2 \times 10^6$  splenocytes ( $n=5$ ) were cultured for up to 14 d at either 3% or 40% oxygen. T-cell-specific activation was achieved by coating plates with CD3 and CD28 antibodies. Telomeric Repeat Amplification Protocol assay was used to quantify telomerase activity in cells isolated at the 3, 5, 7, 10, and 14 d time points.  $n=4$  for each experimental group. **B**, Splenocytes isolated from telomerase reverse transcriptase (TERT)<sup>-/-</sup> mice were cultured for up to 27 d at either 3%, 20%, or 40% oxygen saturation, and total cell numbers were quantified at 2- to 4-d intervals. Dotted line indicates mean cell numbers for wild-type splenocytes after 28 d of cultivation at the same oxygen saturation.  $n=4$  for each experimental group. **C** and **D**, CD4<sup>+</sup> splenocytes from either TERT<sup>+/+</sup> or TERT<sup>-/-</sup> mice were cultured at  $2 \times 10^5$  cells/well under 3% or at 40% oxygen conditions for 14 d. T-cell-specific activation was maintained by addition of antibody-labeled beads at days 0, 7 and 14.  $n=3$  for each experimental group. **E** and **F**, Total splenocytes from either TERT<sup>+/+</sup> or TERT<sup>-/-</sup> mice were cultured at  $1 \times 10^6$  cells/well under 3% in the presence of the telomerase activator TA-65 at indicated concentrations for up to 21 d. T-cell-specific activation was maintained (*Continued*)

$Tert^{-/-}$  splenocytes cultured under physoxic or hyperoxic conditions were observed (Figure 2B). Moreover,  $CD4^{+}$  T-cells immunomagnetically isolated from spleens of F1  $Tert^{-/-}$  mice, opposed to WT  $CD4^{+}$  cells (Figure 2C), also displayed no difference in proliferation rates when cultured in either hyperoxia or physoxia (Figure 2D). To further validate our findings, WT splenocytes were cultured in the presence of the small molecule telomerase activator TA-65.<sup>52</sup> Activation of telomerase in cultured WT splenocytes significantly enhances proliferation in a dose-dependent manner (Figure 2E). As expected, even in the absence of TA-65,  $Tert^{-/-}$  splenocytes demonstrate a decreased baseline proliferation compared with WT cells (Figure 2E and 2F) and failed to respond to TA-65 (Figure 2F). Finally, we investigated the effects of the specific telomerase inhibitor BIBR-1532<sup>53</sup> on splenocyte proliferation at different oxygen saturation levels. Splenocytes demonstrated a dose-dependent attenuation of proliferation when treated with the inhibitor providing further evidence that telomerase activity seems to mediate proliferation when cultured at physoxia (Figure 2G). As expected, splenocytes cultured in hyperoxia demonstrated a reduced proliferation; however, BIBR 1532 treatment resulted in a significant dose-dependent decrease in proliferation at 2 weeks even under these conditions (Figure 2H).

Having demonstrated that oxidative stress suppresses telomerase activity, we next aimed to ascertain whether this reduction in telomerase was at a transcriptional level of *TERT* expression. To investigate the effect of oxidative stress on *mTERT* expression at the single cell level, splenocytes were isolated from telomerase reverse transcriptase reporter mice (*mTert*-GFP) and cultured under the different oxygen conditions (Figure 3A). Under physoxia, ~15% of splenocytes expressed *mTert*-GFP at day 3 and expression decreased thereafter during ongoing cultivation (Figure VIIA and VIIB in the [online-only Data Supplement](#)). In contrast, hyperoxia significantly ( $P<0.0001$ ) attenuated *mTert*-GFP expression in splenocytes at all time points investigated (Figure 3B) demonstrating that oxidative stress inhibits *mTert* expression at a transcriptional level. We next evaluated the total number of *mTert*-GFP expressing cells and the percentage of *mTert*-GFP expressing cells in individual splenocyte subpopulations (Figure 3C and 3D; gating strategy is shown in Figure IV in the [online-only Data Supplement](#)). *mTert*-GFP expression mimicked the proliferative trends of each cell subpopulation in the different oxygen conditions shown in Figure 2.

### Telomerase-Deficient $T_{reg}$ Cells With Long Telomeres Remain Protective Against Atherosclerosis

We have previously established that  $T_{reg}$  cells modulate the development of atherosclerosis in mice. Using adoptive transfer studies, we demonstrated that naturally arising  $T_{reg}$  cells are potent inhibitors of atherosclerosis in several different mouse models.<sup>5</sup> We next examined whether telomerase-deficient

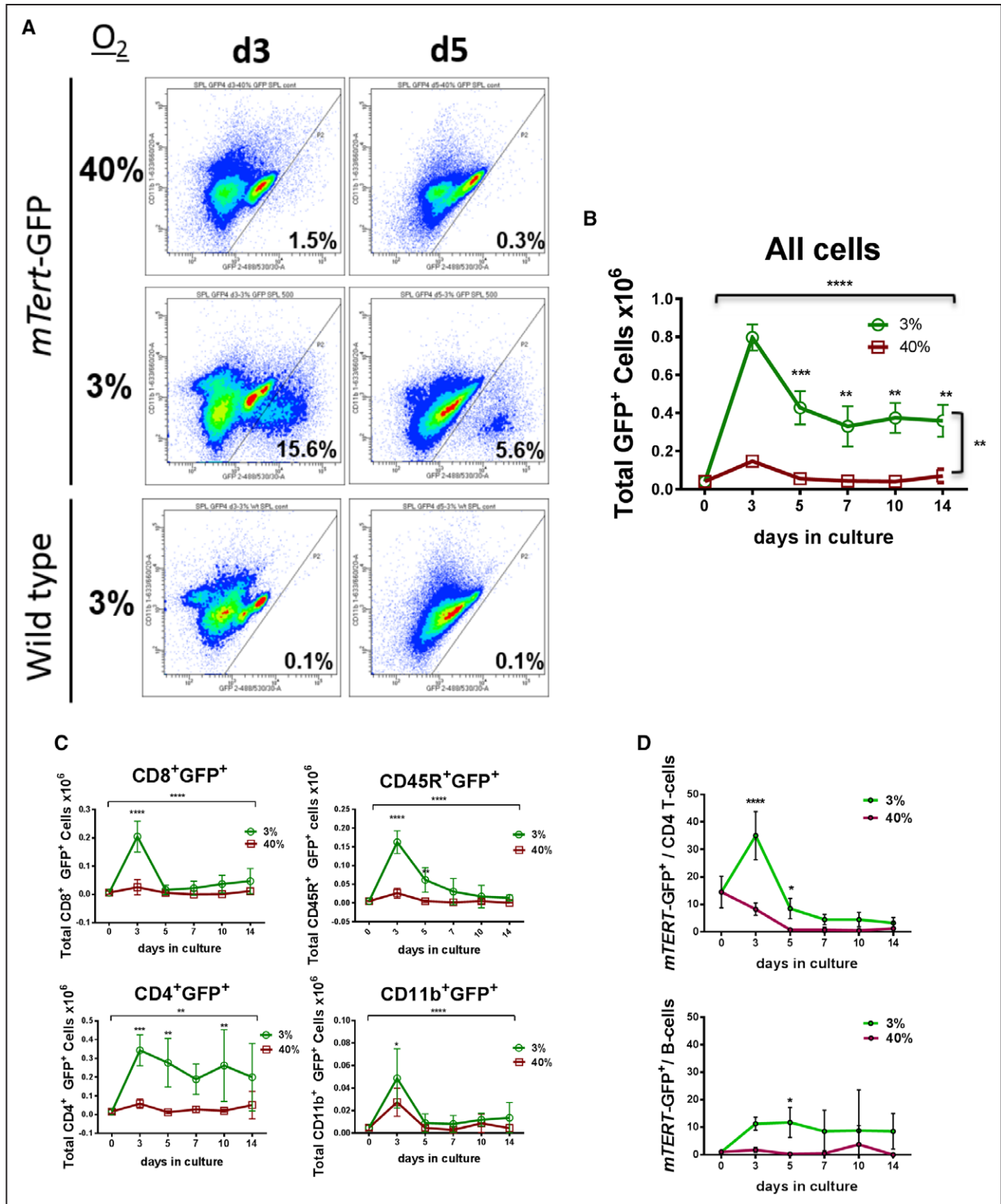
$T_{reg}$  retain the potential to suppress atherosclerosis in vivo. Surprisingly,  $Tert^{-/-}$  mice contained the same number of both  $CD4^{+}$  T-cells and  $T_{reg}$  cells, including the  $CD4^{+}CD25^{+}FoxP3^{+}$  population, as WT mice (Figure 4A–4C). Gating strategy and controls shown in Figure VIIC in the [online-only Data Supplement](#). While acknowledging the potential variation introduced by the digestion protocol, we also quantified the total number of splenocytes obtained from each spleen for WT and  $TERT^{-/-}$  mice. A significant reduction in the total number of cells isolated from  $TERT^{-/-}$  was observed (Figure VIII in the [online-only Data Supplement](#)). To evaluate the functional properties of telomerase-deficient T-cells, we purified  $T_{reg}$  cells and  $CD4^{+}CD25^{-}$  effector T-cells and assessed their proliferative potential ex vivo (representative flow plots are shown in Figure IX in the [online-only Data Supplement](#)).  $T_{reg}$  cells were suppressive of effector T-cells proliferation, regardless of *Tert* expression (Figure 4D and 4E). Having identified that telomerase expression was not required for  $T_{reg}$  suppressive function ex vivo, we investigated the effect of oxidative stress specifically in this population in vitro (Figure 4F). Hyperoxia had no effect on the expansion of the  $CD25^{+} T_{reg}$  population but did significantly diminish proliferation of  $CD25^{-}$  effector T-cells.

To ascertain whether  $TERT$ -deficient  $T_{reg}$  cells remain protective against atherosclerosis in vivo, we adoptively transferred a mixture of  $CD28^{-/-}$  splenocytes, which lack the potential to differentiate into  $T_{reg}$  cells, together with either WT or  $Tert^{-/-}$   $T_{reg}$  into  $Rag2^{-/-}ApoE^{-/-}$  mice, which lack mature lymphocytes. No group of atherogenic Western diet-fed mice displayed any difference in their weight (Figure 5A) and had the same elevated levels of cholesterol (Figure 5B). As expected, total and effector  $CD4^{+}$  T-cells among splenocytes were the same in all 3 groups (Figure 5C) while  $T_{reg}$  were significantly higher in the adoptive transfer groups (15% versus 3%;  $P<0.001$ ; Figure 5C and 5D). The same was true for  $T_{reg}$  content in lymph nodes (25% versus 4%;  $P<0.001$ ; Figure 5D). For all subpopulation analyses, gating strategy and representative flow plots are shown in Figure XA and XB in the [online-only Data Supplement](#). We assessed the atherosclerotic plaque area in the aorta after 7 weeks of Western diet feeding.  $TERT^{-/-} T_{reg}$  were able to suppress plaque area to the same degree as WT  $T_{reg}$  ( $P<0.01$ ; Figure 5E–5G).

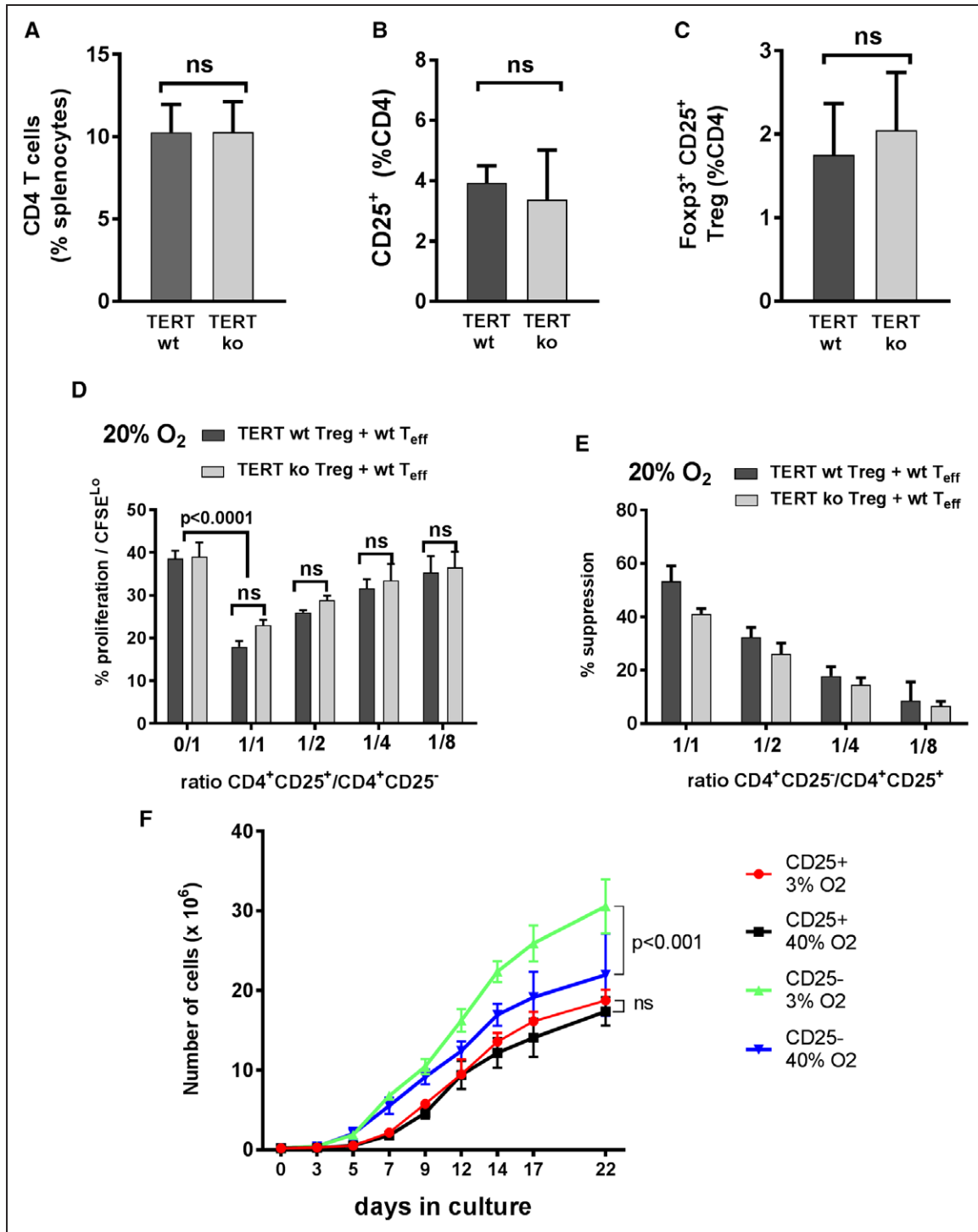
### Telomere Shortening Leads to Reduced Numbers of $T_{reg}$ and Decreases $T_{reg}$ Cell Function

We next aimed to ascertain whether telomere attrition in the  $T_{reg}$  cell population affects their number and function. We have previously demonstrated that bone marrow hematopoietic stem and progenitor cells from 12-month-old first generation (F1)  $TERC^{-/-}$  mice had shorter telomeres compared with WT mice from the same strain (because the heterozygous used to generate  $TERT^{-/-}$  mice already having short telomeres).<sup>62</sup> We compared the percentage of  $CD4^{+}$  T-cells and  $T_{reg}$  in the spleen of both these  $TERC^{-/-}$  and WT mice (Figure 6A and 6B). Genetic knockout of *TERC* did not affect  $CD4^{+}$  T-cell

**Figure 2 Continued.** by antibody-coated plates.  $n=3$  for each experimental group. **G**,  $CD4^{+}$  splenocytes were cultured at  $2 \times 10^5$  cells/well under 3%  $n=3$  for each experimental group, or at **(H)** 40% oxygen conditions for 14 d  $n=4$  for each experimental group. In the presence of BIBR 1532 (telomerase inhibitor) at indicated concentrations, T-cell-specific activation was maintained by addition of antibody-labeled beads (anti-CD3/CD28) at days 0, 7 and 13. All error bars represent the SD. \* $P<0.05$ , \*\*\*\* $P<0.0001$  using with Mann-Whitney *U* test or 2-way ANOVA as appropriate. ns indicates nonsignificant.



**Figure 3.** Oxidative stress suppresses telomerase at the level of *mTert* transcription. Splenocytes were isolated from *mTert*-GFP reporter mice and cultured for 14 d at 3% or 40%, and T-cell activation maintained on antibody-coated plates. *mTert*-GFP (green fluorescent protein) expression was quantified at a single cell level on 3, 4, 7, 10, and 14 d of cultivation by flow cytometry. **A**, Representative flow dot plots for the percentage of total *mTert*-GFP expressing cells at the indicated time points and at both conditions as a percentage of total cells. Gating was established using wild-type mice as controls with <0.1% positive events in the *mTert*-GFP<sup>+</sup> gate. **B**, Absolute numbers of *mTert*-GFP-positive cells within the total splenocyte population over time as quantified by flow cytometry. **C**, Absolute numbers of *mTert*-GFP-positive cells within individual splenocyte subpopulations over time as quantified by flow cytometry. **D**, Percentage of the CD4 (top graph) and B-cell populations (bottom graph) that express *mTert*-GFP at each time point and condition.  $n=3$  for each experimental group. All error bars represent the SD. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , and \*\*\*\* $P<0.0001$  using 2-way ANOVA.

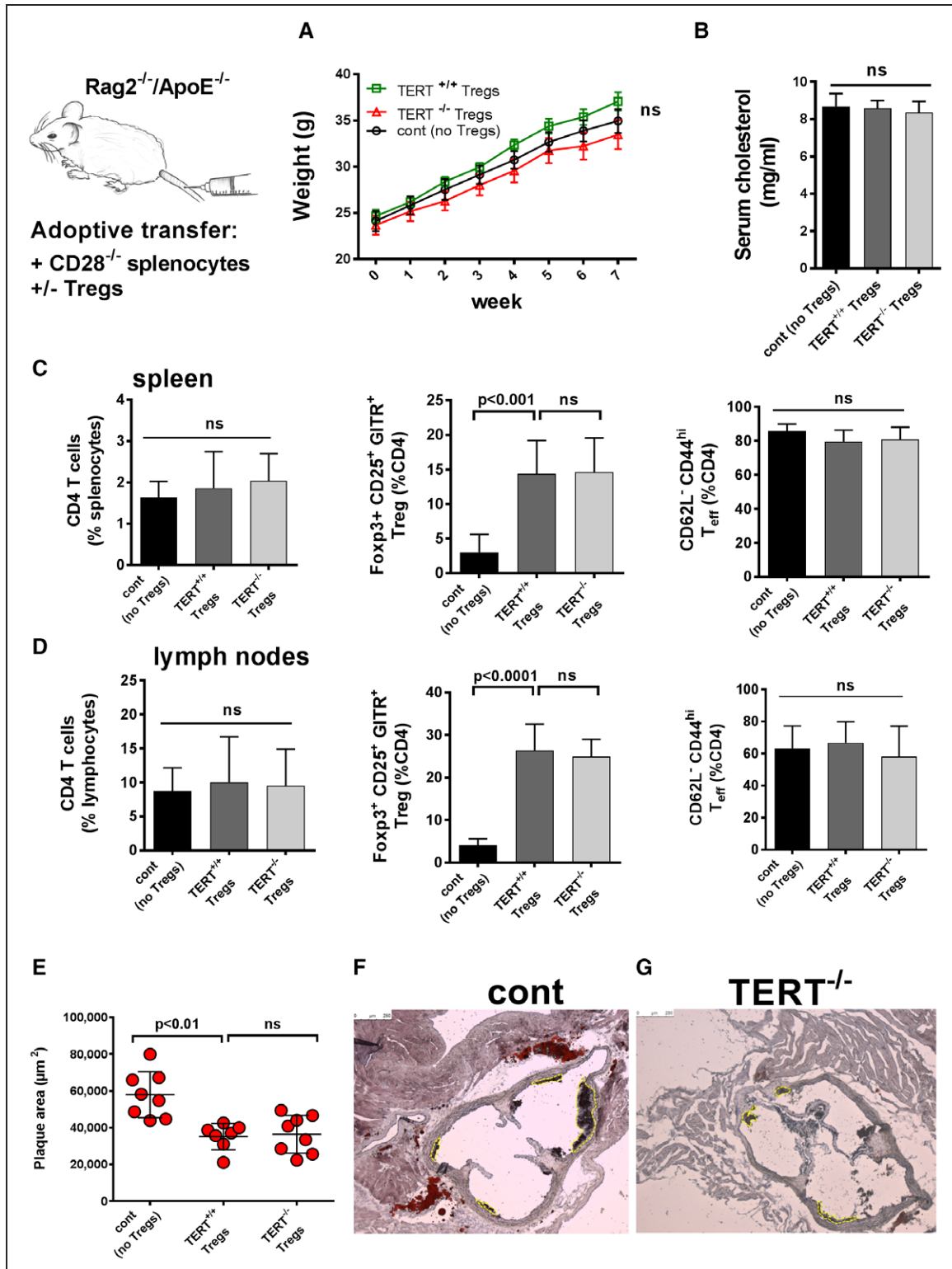


**Figure 4.** Absence of telomerase reverse transcriptase does not affect the in vivo number or suppressive function of regulatory T-cell (Treg) cells in vitro. **A–C**, Flow cytometry was used to quantify CD4<sup>+</sup> and T<sub>reg</sub> cell populations in the spleens of wild-type (WT) or telomerase reverse transcriptase (TERT)<sup>-/-</sup> mice. *n*>4 for each experimental condition. **D** and **E**, CD4<sup>+</sup>CD25<sup>-</sup> effector T-cells (T<sub>eff</sub>) cells were isolated and carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled to allow quantification of proliferation by flow cytometry (CFSE is diluted during each cell division). Coculture with either WT or TERT<sup>-/-</sup> T<sub>reg</sub> cells suppressed T<sub>eff</sub> cell proliferation in a dose-dependent manner. *n*>5 for each experimental condition. **F**, CD25<sup>+/+</sup> cells were isolated from the spleen of WT mice and were cultured for up to 27 d at either 3% or 40% oxygen saturation, and total cell numbers were quantified at indicated intervals. *n*=3 for each experimental condition. Error bars represent SD. A 2-way ANOVA was used for statistical analysis.

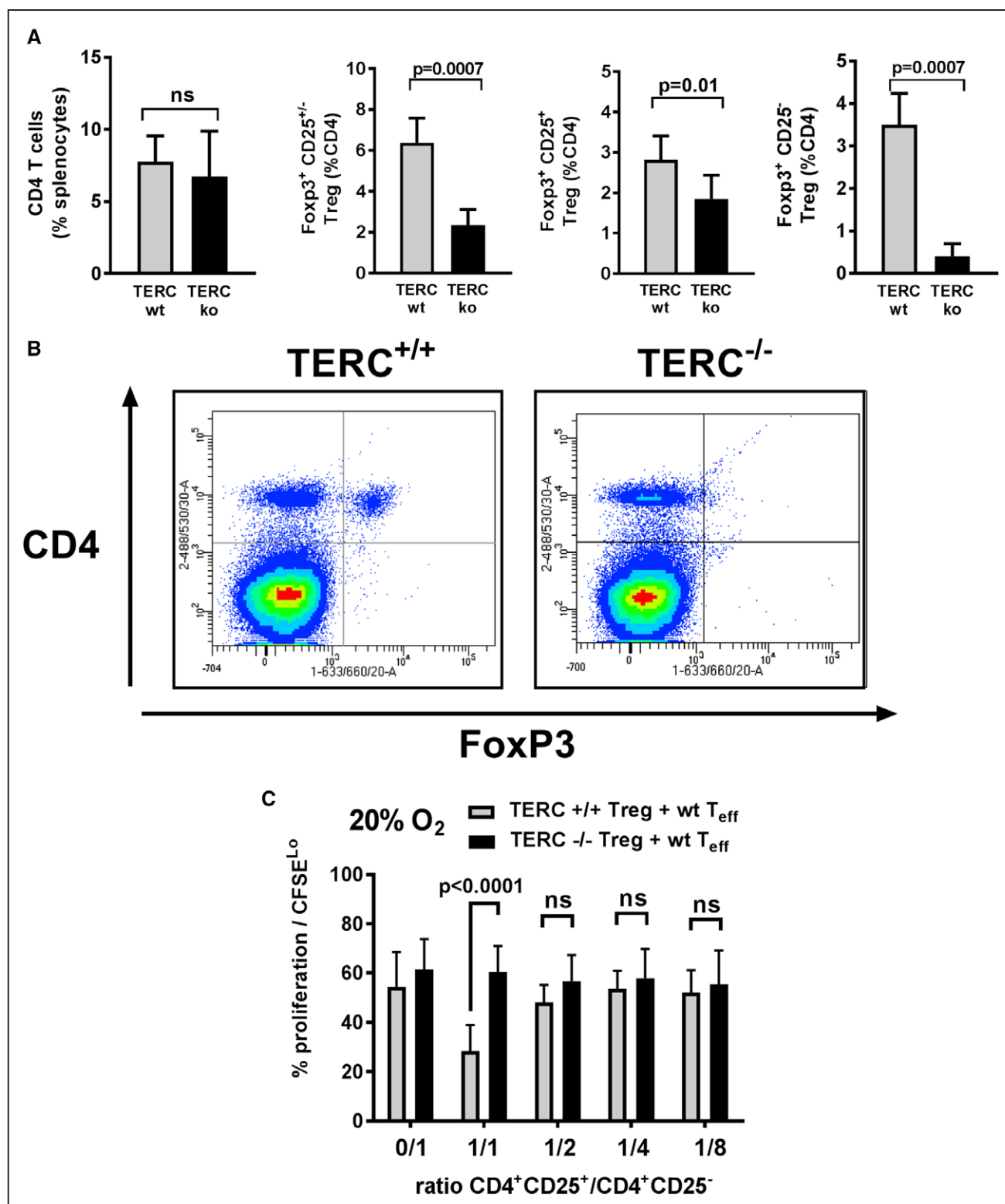
numbers; however, it did result in a significant reduction in the number of T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>; Figure 6B). In addition, we assessed the functional properties of TERC-deficient T<sub>regs</sub>

ex vivo. Surprisingly, unlike TERT-deficient cells (Figure 4D and 4E), TERC<sup>-/-</sup> T<sub>reg</sub> cells lacked suppressive function of effector T-cells proliferation (Figure 6C).





**Figure 5.** Regulatory T-cell ( $T_{reg}$ ) cells lacking telomerase reverse transcriptase remain protective against atherosclerosis. **A**, Rag2<sup>-/-</sup> (recombination activating gene 2) mice were transplanted with telomerase reverse transcriptase (TERT)<sup>-/-</sup>, wild-type (WT)  $T_{reg}$ , or no  $T_{reg}$  (control) and weighed daily. No significant difference in weight was observed between any groups. **B**, Measurements of serum cholesterol. No significant difference was observed between groups. **C** and **D**, No difference in the number of viable CD4<sup>+</sup> or  $T_{reg}$  was observed within the lymph node and spleens of mice transferred with either WT or TERT<sup>-/-</sup>  $T_{reg}$ . Quantification performed by flow cytometry. **E**, Comparison of plaque area between Rag2<sup>-/-</sup> mice transplanted with WT  $T_{reg}$ , TERT<sup>-/-</sup>  $T_{reg}$ , or no  $T_{reg}$ . **F** and **G**, Representative images of plaques stained with Oil Red O in mice transplanted with TERT<sup>-/-</sup>  $T_{reg}$  or no  $T_{reg}$ . Yellow borders highlight plaque area. The data were obtained from 9 mice for each experimental group. Error bars represent SD. A Mann-Whitney U test or 2-way ANOVA was used for statistical analysis as appropriate.



**Figure 6.** Telomerase RNA component (TERC)<sup>-/-</sup> mice have reduced regulatory T-cell (T<sub>reg</sub>) numbers with decreased suppressive function ex vivo. **A**, Flow cytometry was used to quantify CD4<sup>+</sup> and T<sub>reg</sub> cell populations in the spleens of wild-type (WT) or TERC<sup>-/-</sup> mice. n>5 for each experimental condition. **B**, Representative example of flow cytometry plot. **C**, CD4<sup>+</sup>CD25<sup>-</sup> effector T-cells (T<sub>eff</sub>) were isolated and carboxyfluorescein diacetate succinimidyl ester labeled to enable quantification of proliferation by flow cytometry. n=9 for each experimental condition. Coculture with WT (C57BL/6J) but not TERC<sup>-/-</sup> T<sub>reg</sub> cells suppressed T<sub>eff</sub> cell proliferation in a dose-dependent manner. Error bars represent SD. A Mann–Whitney *U* test was used for statistical analysis.

## Discussion

Our data demonstrate that telomerase positively influences T-cell expansion; however, it is not required per se for baseline proliferation or T-cell homeostasis. Evidence suggests that activated T-cells express high levels of telomerase activity to protect their telomeres from accelerated shortening, thereby evading replicative senescence.<sup>63</sup> However, the contribution of telomerase activity to  $T_{reg}$  function had not been investigated to date.

The data we present in this study indicate that telomerase expression is not required for  $T_{reg}$  suppressive function. Although absolute numbers of splenocytes may be decreased, mice with long telomeres that lack functional telomerase activity, like the F1 TERT<sup>-/-</sup> mice used in this study, maintain a  $T_{reg}$  population of relative comparable size to WT animals, which remains functional. Although  $T_{regs}$  express telomerase, it is possible that this cannot indefinitely maintain telomere length, particularly under conditions of oxidative stress that decreases telomerase activity. Interestingly, we have demonstrated that TERC<sup>-/-</sup> mice with shorter haematopoietic stem and progenitor cell telomere length have a severe reduction in peripheral  $T_{reg}$  cell numbers in vivo and impaired function in vitro. This confirms that indeed it is a sufficiently long telomere length rather than the mere presence of telomerase activity that is important for  $T_{reg}$  cell functioning.

For our studies, we have performed adoptive transfer experiments using Rag2<sup>-/-</sup> ApoE<sup>-/-</sup> mice, as previously described.<sup>5</sup> These experiments require the transfer of CD28<sup>-/-</sup> splenocytes which have the potential to differentiate into all lineages of the adaptive immune system except  $T_{reg}$  cells. We are aware that although our study directly tested the functionality of TERT<sup>-/-</sup>  $T_{reg}$  cells in an in vivo disease model, the immune system may be compromised in this model. Further studies are required to determine whether more subtle phenotypes are apparent in alternative nonimmunocompromised models that lack TERT.

Multiple studies have confirmed the association of short leukocyte telomere length and the incidence of CHD in humans.<sup>64</sup> Mendelian randomization studies in >200 000 participants have also suggested that a genetic reduction of telomere length (via mutations in TERT or TERC genes) leads to an increased risk of CHD.<sup>65</sup> Although in our animal studies absence of telomerase had no effect on  $T_{reg}$  maintenance, it is important to point out that because of the long telomeres in laboratory mouse strains, this may not reflect the human biology of telomeres. Indeed, late-generation TERC knockout mice with shorter telomeres are considered more representative of the human situation.

It is known that the adaptive immune system plays a pivotal role in the development and progression of atherosclerosis.<sup>66</sup> Although the proatherogenic function of T-helper type 1 CD4<sup>+</sup> as well as CD8<sup>+</sup> T-cells is well established, it is also emerging that  $T_{reg}$  cells are critical in offsetting the detrimental effects of both adaptive and innate immune responses. What remains unclear is how the plaque environmental milieu influences the net effect of these 2 antagonistic responses and how the molecular mechanisms controlling the survival, proliferation, and suppressive function of the  $T_{reg}$  population contribute to disease progression. We propose that within the

atherosclerotic plaque, chronic oxidative stress conditions may lead to suppression of telomerase and acceleration of telomere attrition in  $T_{regs}$ . This in turn may contribute to premature senescence of  $T_{reg}$  cells and therefore progression of atherosclerosis.

Oxidative stress within the atherosclerosis microenvironment may have other detrimental effects that could contribute to disease progression. Oxidative stress can directly damage both genomic DNA, in the absence of telomere shortening, and mitochondrial DNA, which is linked to vascular senescence and atherosclerosis.<sup>67</sup> Accumulated DNA damage in peripheral blood cells not only occurs in patients with coronary artery disease and acute myocardial infarction<sup>68</sup> but is also associated with outcome.<sup>69</sup> Moreover, high-cholesterol diet and atherosclerosis have been associated with increased DNA damage in peripheral lymphocytes in rabbits.<sup>70</sup> Mitochondrial localization of the TERT protein has been demonstrated to regulate levels of mitochondrial derived reactive oxygen species and TERT can translocate to the mitochondria reducing mitochondrial derived reactive oxygen species production in the process, which is independent of its canonical role in the nucleus.<sup>60</sup> It had been shown in different cell types that lack of TERT specifically in the mitochondria is detrimental to mitochondrial function and morphology.<sup>71</sup> However, such a mitochondrial role has to date not been described in immune cells. We and others have previously demonstrated that TERT can bind to mitochondrial DNA which also protects cells from DNA damage and oxidative stress.<sup>71,72</sup> An antioxidant role of TERT has also recently been described by Beyer et al<sup>53</sup> who have demonstrated that loss of telomerase activity in cells of healthy human vessels results in a switch from nitrous oxide to a proinflammatory hydrogen peroxide, which mediates vascular dilation. Conversely, restoration of telomerase activity in arterioles from humans with coronary artery disease reverts the mediator of flow-induced dilation from hydrogen peroxide to nitrous oxide.<sup>53</sup> The data in our current study demonstrate that oxidative stress attenuates TERT expression at a transcriptional level in lymphocytes. Such reduction in telomerase influences both telomerase nuclear activity and would also diminish some noncanonical functions of telomerase and thereby resistance to oxidative stress further contributing to disease. Indeed, we have demonstrated that oxidative stress and attenuated TERT expression are both associated with an increased production of mitochondrial derived superoxide. We are currently investigating the impact of telomerase activation by TA-65 on canonical (telomere length) and noncanonical (oxidative stress and microvascular endothelial function) pathways in patients with myocardial infarction (TACTIC trial [Telomerase Activator to Reverse Immunosenscence in Acute Coronary Syndrome: A Double-Blind, Phase II, Randomised Controlled Trial]). Noncanonical roles for telomerase may possibly also explain the difference in phenotype between the TERT<sup>-/-</sup> and TERC<sup>-/-</sup> mice in addition to differences in telomere length that are already differentially regulated at the level of heterozygotes in both knockout models.<sup>73</sup> The TERC<sup>-/-</sup> mice might still benefit from noncanonical roles of telomerase because of the presence of TERT.

Correlative evidence from human population studies collectively suggests an association of short telomeres in

lymphocytes with conditions of increased oxidative stress, including smoking, obesity, and CHD.<sup>27,28,32,33,74–76</sup> Furthermore, nonimmune cells have been shown to have accelerated telomere attrition and develop premature senescence when cultured under hyperoxic conditions.<sup>18,47</sup> This could represent one mechanism that would explain why patients with short telomeres in lymphocytes are predisposed to CHD, a hypothesis supported by our recent work demonstrating that immunosenescence can be an independent predictor of disease outcome in older people, possibly via accelerated atherosclerosis.<sup>77</sup>

The only study showing an atheroprotective effect of global telomerase deficiency as well as shortened telomeres in the ApoE mouse model<sup>38</sup> may be explained by global immunosenescence and a dysfunctional immune system, where the lack of myeloid cell proliferation is responsible for reduced plaque progression. Of note, our experiments demonstrate that in contrast to T-cells, proliferation of myeloid cells was indeed not influenced by oxidative stress. Therefore, the plaque environment seems to modulate the local immune response in a manner conducive for chronic inflammation. A dichotomous protective versus pathological role of telomerase as a result of tissue specificity has already been described (reviewed in 78). Telomerase activation has been demonstrated to reduce reactive oxygen species production and thereby inflammation in the endothelium whereas increased telomerase activity within the vascular smooth muscle layer can result in abnormal proliferation and vascular remodeling in hypertensive rats.<sup>78</sup>

Finally, even in light of recent studies demonstrating that critically short telomeres can be protective for atherosclerosis, our current data suggest that restoring telomerase activity in T-cells, for example, through small molecule activators such as TA-65,<sup>52,79</sup> remains a potentially powerful therapeutic intervention.

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## Disclosures

None.

## References

- Taleb S, Tedgui A, Mallat Z. Adaptive T cell immune responses and atherogenesis. *Curr Opin Pharmacol*. 2010;10:197–202. doi: 10.1016/j.coph.2010.02.003.
- Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A. Protective role of interleukin-10 in atherosclerosis. *Circ Res*. 1999;85:e17–e24.
- Taleb S, Tedgui A, Mallat Z. Regulatory T-cell immunity and its relevance to atherosclerosis. *J Intern Med*. 2008;263:489–499. doi: 10.1111/j.1365-2796.2008.01944.x.
- Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. The role of adaptive T cell immunity in atherosclerosis. *J Lipid Res*. 2009;50(suppl):S364–S369. doi: 10.1194/jlr.R800092-JLR200.
- Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D, Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med*. 2006;12:178–180. doi: 10.1038/nm1343.
- Sasaki N, Yamashita T, Takeda M, Shinohara M, Nakajima K, Tawa H, Usui T, Hirata K. Oral anti-CD3 antibody treatment induces regulatory T cells and inhibits the development of atherosclerosis in mice. *Circulation*. 2009;120:1996–2005. doi: 10.1161/CIRCULATIONAHA.109.863431.
- Mor A, Planer D, Luboshits G, Afek A, Metzger S, Chajek-Shaul T, Keren G, George J. Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:893–900. doi: 10.1161/01.ATV.0000259365.31469.89.
- Entin-Meer M, Afek A, George J. Regulatory T-cells, FoxP3 and atherosclerosis. *Adv Exp Med Biol*. 2009;665:106–114.
- Lin J, Li M, Wang Z, He S, Ma X, Li D. The role of CD4+CD25+ regulatory T cells in macrophage-derived foam-cell formation. *J Lipid Res*. 2010;51:1208–1217. doi: 10.1194/jlr.D000497.
- Foks AC, Frodermann V, ter Borg M, Habets KL, Bot I, Zhao Y, van Eck M, van Berkel TJ, Kuiper J, van Puijvelde GH. Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis*. 2011;218:53–60. doi: 10.1016/j.atherosclerosis.2011.04.029.
- Weber C, Meiler S, Döring Y, et al. CCL17-expressing dendritic cells drive atherosclerosis by restraining regulatory T cell homeostasis in mice. *J Clin Invest*. 2011;121:2898–2910. doi: 10.1172/JCI44925.
- Maganto-García E, Tarrio ML, Grabie N, Bu DX, Lichtman AH. Dynamic changes in regulatory T cells are linked to levels of diet-induced hypercholesterolemia. *Circulation*. 2011;124:185–195. doi: 10.1161/CIRCULATIONAHA.110.006411.
- Ait-Oufella H, Kinugawa K, Zoll J, et al. Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. *Circulation*. 2007;115:2168–2177. doi: 10.1161/CIRCULATIONAHA.106.662080.
- Taleb S, Herbin O, Ait-Oufella H, Verreth W, Gourdy P, Barateau V, Merval R, Esposito B, Clément K, Holvoet P, Tedgui A, Mallat Z. Defective leptin/leptin receptor signaling improves regulatory T cell immune response and protects mice from atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:2691–2698. doi: 10.1161/ATVBAHA.107.149567.
- Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC, Akbar AN. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest*. 2006;116:2423–2433. doi: 10.1172/JCI28941.
- Weng NP, Levine BL, June CH, Hodes RJ. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc Natl Acad Sci USA*. 1995;92:11091–11094.
- Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. *J Mol Biol*. 1992;225:951–960.
- von Zglinicki T, Saretzki G, Döcke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Exp Cell Res*. 1995;220:186–193. doi: 10.1006/excr.1995.1305.
- Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA. Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell*. 1999;96:701–712.
- Herrera E, Samper E, Martín-Caballero J, Flores JM, Lee HW, Blasco MA. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. *EMBO J*. 1999;18:2950–2960. doi: 10.1093/emboj/18.11.2950.
- Meznikova M, Erdmann N, Allsopp R, Harrington LA. Telomerase reverse transcriptase-dependent telomere equilibration mitigates tissue dysfunction in mTert heterozygotes. *Dis Model Mech*. 2009;2:620–626. doi: 10.1242/dmm.004069.
- Roth A, Yssel H, Pene J, Chavez EA, Schertzer M, Lansdorp PM, Spits H, Luiten RM. Telomerase levels control the lifespan of human T lymphocytes. *Blood*. 2003;102:849–857. doi: 10.1182/blood-2002-07-2015.
- Choudhury AR, Ju Z, Djojicubroto MW, et al. Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. *Nat Genet*. 2007;39:99–105. doi: 10.1038/ng1937.
- Blasco MA. Immunosenescence phenotypes in the telomerase knockout mouse. *Springer Semin Immunopathol*. 2002;24:75–85.



25. Petersen S, Saretzki G, von Zglinicki T. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res*. 1998;239:152–160.
26. Hewitt G, Jurk D, Marques FD, Correia-Melo C, Hardy T, Gackowska A, Anderson R, Taschuk M, Mann J, Passos JF. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat Commun*. 2012;3:708. doi: 10.1038/ncomms1708.
27. Samani NJ, Erdmann J, Hall AS, et al; WTCCC and the Cardiogenics Consortium. Genome-wide association analysis of coronary artery disease. *N Engl J Med*. 2007;357:443–453. doi: 10.1056/NEJMoa072366.
28. Brouillette SW, Whittaker A, Stevens SE, van der Harst P, Goodall AH, Samani NJ. Telomere length is shorter in healthy offspring of subjects with coronary artery disease: support for the telomere hypothesis. *Heart*. 2008;94:422–425. doi: 10.1136/hrt.2007.139675.
29. Samani NJ, Boulthby R, Butler R, Thompson JR, Goodall AH. Telomere shortening in atherosclerosis. *Lancet*. 2001;358:472–473. doi: 10.1016/S0140-6736(01)05633-1.
30. Brouillette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol*. 2003;23:842–846. doi: 10.1161/01.ATV.0000067426.96344.32.
31. Brouillette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, Packard CJ, Samani NJ; West of Scotland Coronary Prevention Study Group. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. *Lancet*. 2007;369:107–114. doi: 10.1016/S0140-6736(07)60071-3.
32. Farzaneh-Far R, Cawthon RM, Na B, Browner WS, Schiller NB, Whooley MA. Prognostic value of leukocyte telomere length in patients with stable coronary artery disease: data from the Heart and Soul Study. *Arterioscler Thromb Vasc Biol*. 2008;28:1379–1384. doi: 10.1161/ATVBAHA.108.167049.
33. van der Harst P, van der Steege G, de Boer RA, Voors AA, Hall AS, Mulder MJ, van Gilst WH, van Veldhuisen DJ; MERIT-HF Study Group. Telomere length of circulating leukocytes is decreased in patients with chronic heart failure. *J Am Coll Cardiol*. 2007;49:1459–1464. doi: 10.1016/j.jacc.2007.01.027.
34. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature*. 2010;464:520–528. doi: 10.1038/nature08982.
35. Nzietchueng R, Elfarra M, Nloga J, Labat C, Carteaux JP, Maureira P, Lacomblet P, Villemot JP, Benetos A. Telomere length in vascular tissues from patients with atherosclerotic disease. *J Nutr Health Aging*. 2011;15:153–156.
36. Toupance S, Labat C, Temmar M, Rossignol P, Kimura M, Aviv A, Benetos A. Short telomeres, but not telomere attrition rates, are associated with carotid atherosclerosis. *Hypertension*. 2017;70:420–425. doi: 10.1161/HYPERTENSIONAHA.117.09354.
37. Needham BL, Rehkopf D, Adler N, Gregorich S, Lin J, Blackburn EH, Epel ES. Leukocyte telomere length and mortality in the National Health and Nutrition Examination Survey, 1999–2002. *Epidemiology*. 2015;26:528–535. doi: 10.1097/EDE.0000000000000299.
38. Poch E, Carbonell P, Franco S, Díez-Juan A, Blasco MA, Andrés V. Short telomeres protect from diet-induced atherosclerosis in apolipoprotein E-null mice. *FASEB J*. 2004;18:418–420. doi: 10.1096/fj.03-0710fje.
39. Cortés MJ, Díez-Juan A, Pérez P, Pérez-Roger I, Arroyo-Pellicer R, Andrés V. Increased early atherogenesis in young versus old hypercholesterolemic rabbits by a mechanism independent of arterial cell proliferation. *FEBS Lett*. 2002;522:99–103.
40. Fernández-Alvira JM, Fuster V, Dorado B, Soberón N, Flores I, Gallardo M, Pocock S, Blasco MA, Andrés V. Short telomere load, telomere length, and subclinical atherosclerosis: the PESA Study. *J Am Coll Cardiol*. 2016;67:2467–2476. doi: 10.1016/j.jacc.2016.03.530.
41. Bischoff C, Petersen HC, Graakjaer J, Andersen-Ranberg K, Vaupel JW, Bohr VA, Kølvrå S, Christensen K. No association between telomere length and survival among the elderly and oldest old. *Epidemiology*. 2006;17:190–194. doi: 10.1097/01.ede.0000199436.55248.10.
42. Sanders JL, Newman AB. Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? *Epidemiol Rev*. 2013;35:112–131. doi: 10.1093/epirev/mxs008.
43. Martin-Ruiz CM, Gussekloo J, van Heemst D, von Zglinicki T, Westendorp RG. Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population-based study. *Aging Cell*. 2005;4:287–290. doi: 10.1111/j.1474-9726.2005.00171.x.
44. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet*. 2003;361:393–395. doi: 10.1016/S0140-6736(03)12384-7.
45. Chiang YJ, Hemann MT, Hattcock KS, Tessarollo L, Feigenbaum L, Hahn WC, Hodes RJ. Expression of telomerase RNA template, but not telomerase reverse transcriptase, is limiting for telomere length maintenance in vivo. *Mol Cell Biol*. 2004;24:7024–7031. doi: 10.1128/MCB.24.16.7024-7031.2004.
46. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorf PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*. 1997;91:25–34.
47. Bennaceur K, Atwill M, Al Zhrany N, Hoffmann J, Keavney B, Breault D, Richardson G, von Zglinicki T, Saretzki G, Spyridopoulos I. Atorvastatin induces T cell proliferation by a telomerase reverse transcriptase (TERT) mediated mechanism. *Atherosclerosis*. 2014;236:312–320. doi: 10.1016/j.atherosclerosis.2014.07.020.
48. Breault DT, Min IM, Carlone DL, Farilla LG, Ambruzs DM, Henderson DE, Algra S, Montgomery RK, Wagers AJ, Hole N. Generation of mTert-GFP mice as a model to identify and study tissue progenitor cells. *Proc Natl Acad Sci USA*. 2008;105:10420–10425. doi: 10.1073/pnas.0804800105.
49. Montgomery RK, Carlone DL, Richmond CA, Farilla L, Kranendonk ME, Henderson DE, Baffour-Awuah NY, Ambruzs DM, Fogli LK, Algra S, Breault DT. Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proc Natl Acad Sci USA*. 2011;108:179–184. doi: 10.1073/pnas.1013004108.
50. Richardson GD, Breault D, Horrocks G, Cormack S, Hole N, Owens WA. Telomerase expression in the mammalian heart. *FASEB J*. 2012;26:4832–4840. doi: 10.1096/fj.12-208843.
51. Song J, Czerniak S, Wang T, Ying W, Carlone DL, Breault DT, Humphreys BD. Characterization and fate of telomerase-expressing epithelia during kidney repair. *J Am Soc Nephrol*. 2011;22:2256–2265. doi: 10.1681/ASN.2011050447.
52. Bernardes de Jesus B, Schneeberger K, Vera E, Tejera A, Harley CB, Blasco MA. The telomerase activator TA-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence. *Aging Cell*. 2011;10:604–621.
53. Beyer AM, Freed JK, Durand MJ, Riedel M, Ait-Aissa K, Green P, Hockenberry JC, Morgan RG, Donato AJ, Peleg R, Gasparri M, Rokkas CK, Santos JH, Priel E, Gutterman DD. Critical role for telomerase in the mechanism of flow-mediated dilation in the human microcirculation. *Circ Res*. 2016;118:856–866. doi: 10.1161/CIRCRESAHA.115.307918.
54. Owusu-Ansah E, Yavari A, Banerjee U. A protocol for in vivo detection of reactive oxygen species. *Protocol Exchange*. February 27, 2008. doi:10.1038/nprot.2008.23. <https://www.nature.com/protocolexchange/protocols/414>.
55. Mukhopadhyay P, Rajesh M, Haskó G, Hawkins BJ, Madesh M, Pacher P. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nat Protoc*. 2007;2:2295–2301. doi: 10.1038/nprot.2007.327.
56. Ashor AW, Chowdhury S, Oggioni C, Qadir O, Brandt K, Ishaq A, Mathers JC, Saretzki G, Siervo M. Inorganic nitrate supplementation in young and old obese adults does not affect acute glucose and insulin responses but lowers oxidative stress. *J Nutr*. 2016;146:2224–2232.
57. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res*. 2001;89:E41–E45.
58. von Zglinicki T. Oxidative stress shortens telomeres. *Trends Biochem Sci*. 2002;27:339–344.
59. Saretzki G, Von Zglinicki T. Replicative aging, telomeres, and oxidative stress. *Ann NY Acad Sci*. 2002;959:24–29.
60. Ahmed S, Passos JF, Birket MJ, Beckmann T, Brings S, Peters H, Birch-Machin MA, von Zglinicki T, Saretzki G. Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. *J Cell Sci*. 2008;121(pt 7):1046–1053. doi: 10.1242/jcs.019372.
61. Meyerson M, Counter CM, Eaton EN, Ellison LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell*. 1997;90:785–795.
62. Al-Ajmi N, Saretzki G, Miles C, Spyridopoulos I. Dietary restriction ameliorates haematopoietic ageing independent of telomerase, whilst lack of telomerase and short telomeres exacerbates the ageing phenotype. *Exp Gerontol*. 2014;58:113–119. doi: 10.1016/j.exger.2014.07.010.
63. Weng NP, Levine BL, June CH, Hodes RJ. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J Exp Med*. 1996;183:2471–2479.

64. Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A, Willeit P. Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. *BMJ*. 2014;349:g4227.
65. Zhan Y, Karlsson IK, Karlsson R, Tillander A, Reynolds CA, Pedersen NL, Hägg S. Exploring the causal pathway from telomere length to coronary heart disease: a Network Mendelian Randomization Study. *Circ Res*. 2017;121:214–219. doi: 10.1161/CIRCRESAHA.116.310517.
66. Gotsman I, Grabie N, Dacosta R, Sukhova G, Sharpe A, Lichtman AH. Proatherogenic immune responses are regulated by the PD-1/PD-L pathway in mice. *J Clin Invest*. 2007;117:2974–2982. doi: 10.1172/JCI31344.
67. Borghini A, Cervelli T, Galli A, Andreassi MG. DNA modifications in atherosclerosis: from the past to the future. *Atherosclerosis*. 2013;230:202–209. doi: 10.1016/j.atherosclerosis.2013.07.038.
68. Demirbag R, Yilmaz R, Gur M, Kocyigit A, Celik H, Guzel S, Selek S. Lymphocyte DNA damage in patients with acute coronary syndrome and its relationship with severity of acute coronary syndrome. *Mutat Res*. 2005;578:298–307. doi: 10.1016/j.mrfmmm.2005.05.005.
69. Federici C, Botto N, Manfredi S, Rizza A, Del Fiandra M, Andreassi MG. Relation of increased chromosomal damage to future adverse cardiac events in patients with known coronary artery disease. *Am J Cardiol*. 2008;102:1296–1300. doi: 10.1016/j.amjcard.2008.07.024.
70. Ramirez-Tortosa MC, Granados S, Ramirez-Tortosa CL, Ochoa JJ, Camacho P, García-Valdés L, Battino M, Quiles JL. Oxidative stress status in liver mitochondria and lymphocyte DNA damage of atherosclerotic rabbits supplemented with water soluble coenzyme Q10. *Biofactors*. 2008;32:263–273.
71. Sharma NK, Reyes A, Green P, Caron MJ, Bonini MG, Gordon DM, Holt JJ, Santos JH. Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria. *Nucleic Acids Res*. 2012;40:712–725. doi: 10.1093/nar/gkr758.
72. Haendeler J, Dröse S, Büchner N, Jakob S, Altschmied J, Goy C, Spyridopoulos I, Zeiher AM, Brandt U, Dimmeler S. Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. *Arterioscler Thromb Vasc Biol*. 2009;29:929–935. doi: 10.1161/ATVBAHA.109.185546.
73. Hathcock KS, Hemann MT, Opperman KK, Strong MA, Greider CW, Hodes RJ. Haploinsufficiency of mTR results in defects in telomere elongation. *Proc Natl Acad Sci USA*. 2002;99:3591–3596. doi: 10.1073/pnas.012549799.
74. Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. Accelerated telomere shortening in response to life stress. *Proc Natl Acad Sci USA*. 2004;101:17312–17315. doi: 10.1073/pnas.0407162101.
75. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. *Lancet*. 2005;366:662–664. doi: 10.1016/S0140-6736(05)66630-5.
76. Spyridopoulos I, Hoffmann J, Aicher A, Brummendorf TH, Doerr HW, Zeiher AM, Dimmeler S. Accelerated telomere shortening in leukocyte subpopulations of patients with coronary heart disease: role of cytomegalovirus seropositivity. *Circulation*. 2009;120:1364–1372. doi: 10.1161/CIRCULATIONAHA.109.854299.
77. Spyridopoulos I, Martin-Ruiz C, Hilken C, Yadegarfar ME, Isaacs J, Jagger C, Kirkwood T, von Zglinicki T. CMV seropositivity and T-cell senescence predict increased cardiovascular mortality in octogenarians: results from the Newcastle 85+ study. *Aging Cell*. 2016;15:389–392. doi: 10.1111/accel.12430.
78. Ait-Aissa K, Ebben JD, Kadlec AO, Beyer AM. Friend or foe? Telomerase as a pharmacological target in cancer and cardiovascular disease. *Pharmacol Res*. 2016;111:422–433. doi: 10.1016/j.phrs.2016.07.003.
79. Salvador L, Singaravelu G, Harley CB, Flom P, Suram A, Raffaele JM. A natural product telomerase activator lengthens telomeres in humans: a Randomized, Double Blind, and Placebo Controlled Study. *Rejuvenation Res*. 2016;19:478–484. doi: 10.1089/rej.2015.1793.

### Highlights

- Hyperoxia induces oxidative stress and T-cell activation and reduced CD4<sup>+</sup> T-cell proliferation in vitro.
- Oxidative stress-mediated reduction in CD4<sup>+</sup> T-cell proliferation is telomerase reverse transcriptase dependent.
- Telomerase reverse transcriptase deficiency has no effect on regulatory T-cell (T<sub>reg</sub>) numbers in vivo or suppressive function of the T<sub>reg</sub> population ex vivo.
- Oxidative stress had no effect on the proliferation of the T<sub>reg</sub> subpopulation or CD11b<sup>+</sup> myeloid cells in vitro.
- Telomerase function was not required for the ability of T<sub>regs</sub> to protect against atherosclerosis in vivo; however, telomere length is critical for T<sub>reg</sub> function.

# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

## **Telomerase Mediates Lymphocyte Proliferation but Not the Atherosclerosis-Suppressive Potential of Regulatory T-Cells**

Gavin David Richardson, Andrew Sage, Karim Bennaceur, Nayef Al Zhrany, Jose Coelho-Lima, Emily Dookun, Lilia Draganova, Gabriele Saretzki, David T. Breault, Ziad Mallat and Ioakim Spyridopoulos

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## Major Resources Tables

### Animals

Species/Strain	Vendor or Source	Background Strain	Sex
Wild type	Charles river	C57BL/6J	Male and Female
B6.129S-Tert, tm1Yjc/J (TERT <sup>-/-</sup> )	Jackson Laboratory	C57BL/6J	Male and Female
Cg-Terc tm1Rdp/J (TERC <sup>-/-</sup> )	Jackson Laboratory	C57BL/6J	Male and Female
<i>mTert</i> -GFP	David Breault Harvard.	C57BL/6J. 129S	Male and Female
Rag2 <sup>-/-</sup> ApoE <sup>-/-</sup> double knock out	Charlies River	C57BL/6J	Male and Female
CD28 <sup>-/-</sup>	Charlies River	C57BL/6J	Male and Female

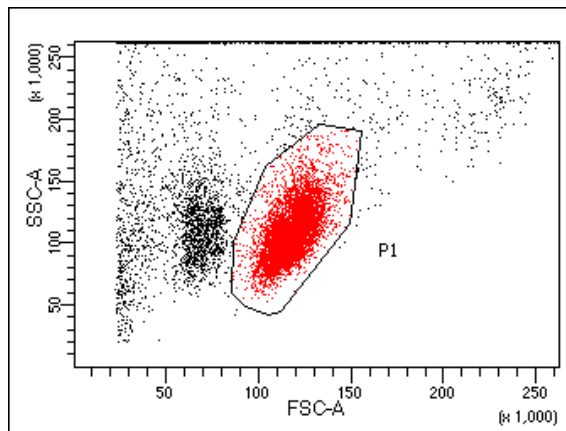
### Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
anti-CD4 (L3T4). For Isolation	Miltenyi Biotec	130-049-201	As per manufacture instructions	
MACSibead mouse T-cell expansion beads	Miltenyi Biotec	130-093-627	As per manufacture instructions	
Anti CD-3. For T cell activation	BD Biosciences	BD 553238	0.5ug/ml	
Anti CD-28. For T cell activation	BD Biosciences	BD 553295	0.5ug/ml	
CD45R (PerCP)	BD Biosciences	553093	0.1 µg/ml	
CD11b (APC)	BD Biosciences	561690	0.1 µg/ml	
CD4 (PE-Cy)	BD Biosciences	552775	0.1 µg/ml	
CD8 (Pacific Blue)	BD Biosciences	558106	0.1 µg/ml	
T <sub>reg</sub> Detection Kit	Miltenyi Biotec	130-094-164	As per manufacture instructions	
CD4 <sup>+</sup> CD25 <sup>+</sup> T <sub>reg</sub> isolation kit	Miltenyi Biotec	130-091-041	As per manufacture instructions	
CD4	Biolegend	100531	1µg/ml	
CD25	Biolegend	102012	1µg/ml	
CD3	Biolegend	100321	2µg/ml	
CD62L	Biolegend	104424	2.5µg/ml	
CD44	Biolegend	103012	0.5µg/ml	
Foxp3	Life Technologies	12-5773-82	2µg/ml	

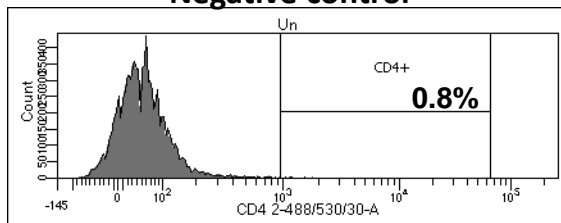


## SUPPLEMENTAL MATERIAL

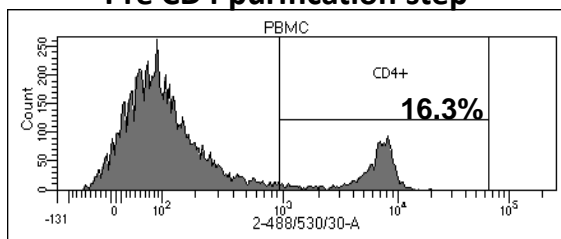
### Supplementary Figure I



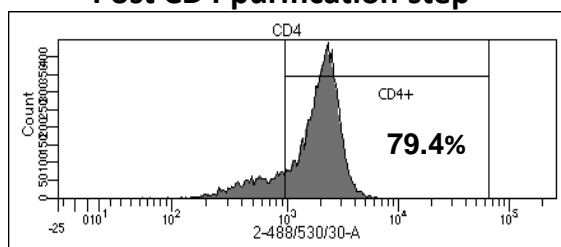
#### Negative control



#### Pre CD4 purification step

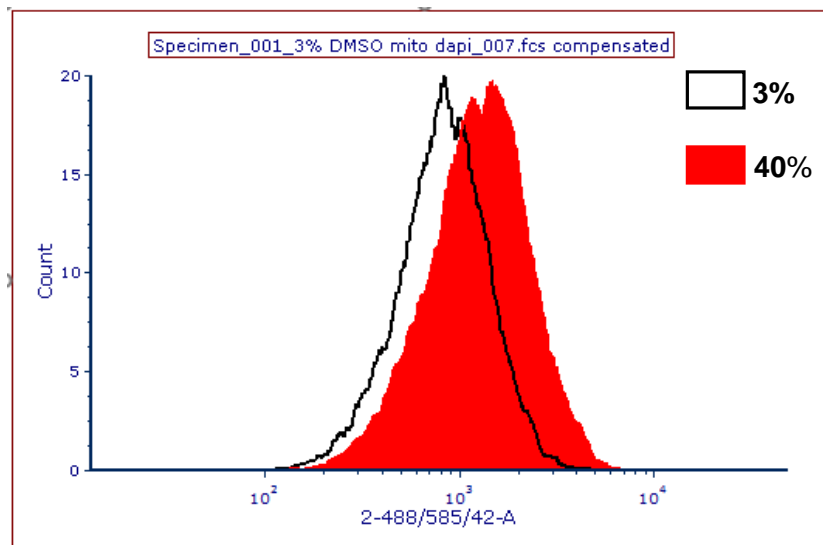


#### Post CD4 purification step



**Supplementary Figure I. Example of assessment of CD4 cell purity after isolation CD4 magnetic sorting.**

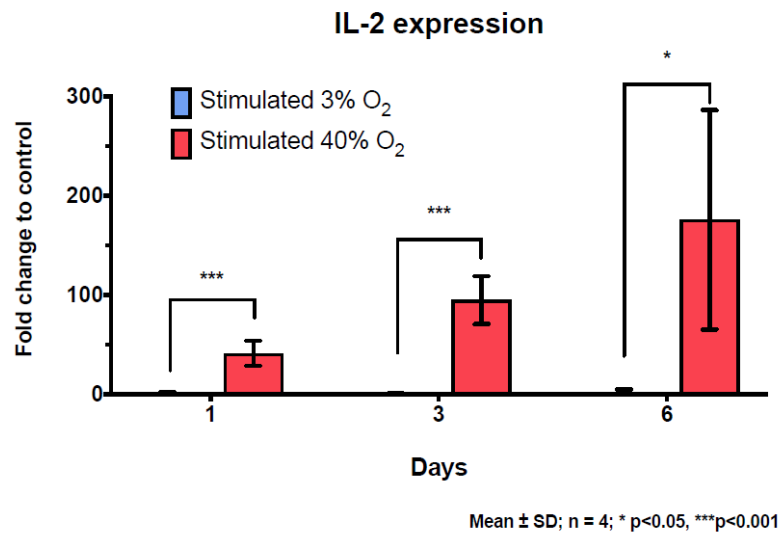
## Supplementary Figure II



**Supplementary Figure II. Hyperoxic culture leads to increased mitochondrial superoxide production.** Total splenocytes were cultured at either 3% or 40% oxygen saturation for 6 days. Mitosox fluorescence was increased under hyperoxia compared to cells cultured under 3% oxygen as indicated by a shift in the mean and geomean values when analysed by flow cytometry.



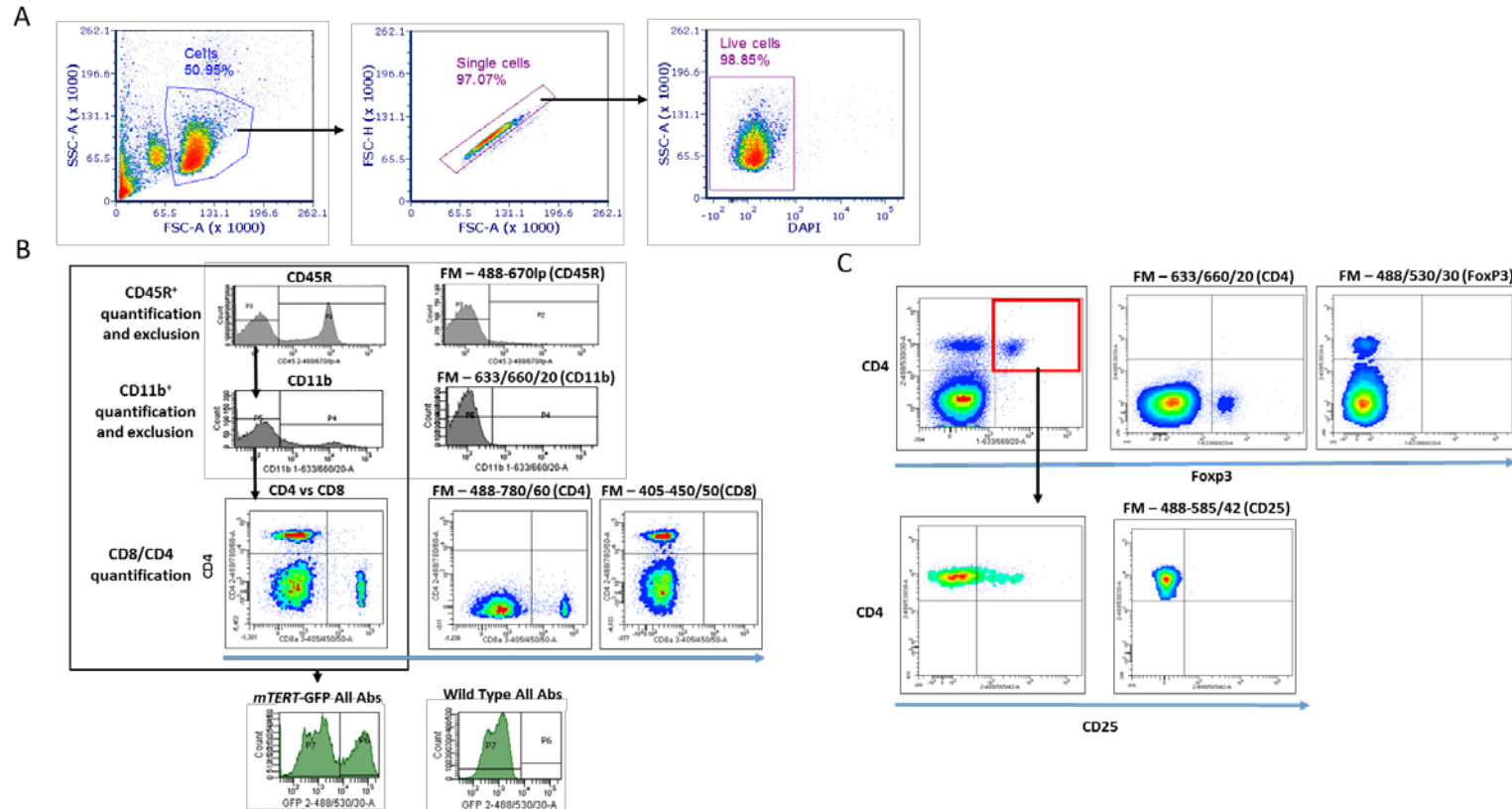
### Supplementary Figure III



#### **Supplementary Figure III. Oxidative stress leads to increased IL2 expression.**

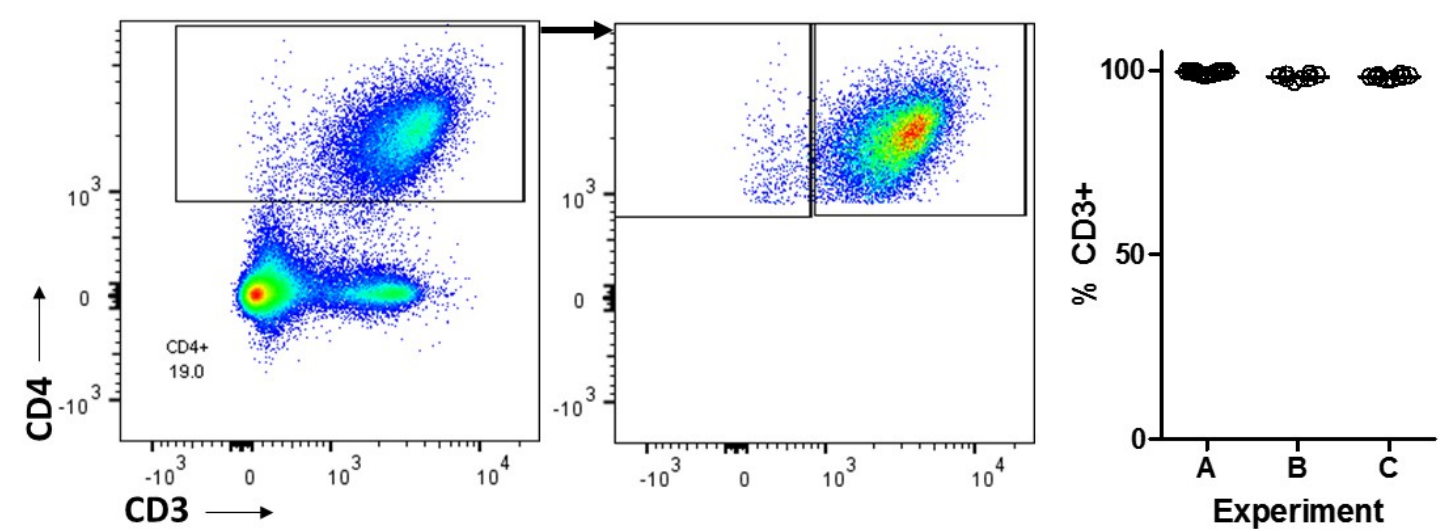
CD4<sup>+</sup> splenocytes were cultured at  $2 \times 10^5$  cells/well under 3% or at 40% oxygen conditions for up to 6 days. IL2 transcript expression was significantly increased at 1, 3 and 6 days under hyperoxia as quantified by qRT-PCR. All error bars represent the standard deviation N>4 for each experimental condition. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 and \*\*\*\*P<0.0001 using A 2-WAY ANOVA.

## Supplementary Figure IV



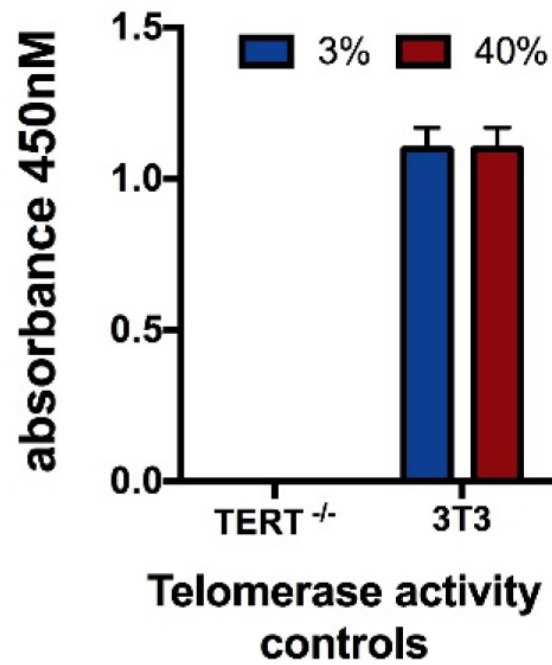
**Supplementary Figure IV. Gating strategies.** **A)** Cells were first discriminated from debris based on SSC vs FSC. FSC-H vs FSC-A was used to establish a singlet gate. Cells within the singlet gate were assessed for viability (DAPI exclusion). **B)** Gating strategy for quantification of CD4<sup>+</sup>/CD8<sup>+</sup> T-cells, B cells (CD45R<sup>+</sup>), myeloid cells (CD11b<sup>+</sup>) and *mTert*-GFP. Quantification of subpopulations performed as shown. Gating set to fluorescence minus one controls (FMO). In some experiments *mTert*-GFP was quantified within each subpopulation following the initial gating. Wild type splenocytes stained with all antibodies were used to establish positive gate for GFP expression. **C)** Quantification of T<sub>reg</sub> populations isolated directly from spleens (As in Figure 4 A-C and Figure 6A). CD4<sup>+</sup>CD8<sup>+</sup> cells were identified using fluorescence minus one (FMO) controls to set gating. For these plots the heading indicates the channel in which the absent fluorophore would have been detected and the name of the absent antibody. CD25<sup>+</sup>-, CD25<sup>+</sup> and CD25<sup>-</sup> were quantified as a percentage of the CD4<sup>+</sup>CD8<sup>+</sup> population.

Supplementary Figure V.



Supplementary Figure V. Quantification of the percentage of CD3<sup>+</sup> T-Cells in the CD4<sup>+</sup> population in mouse spleen.

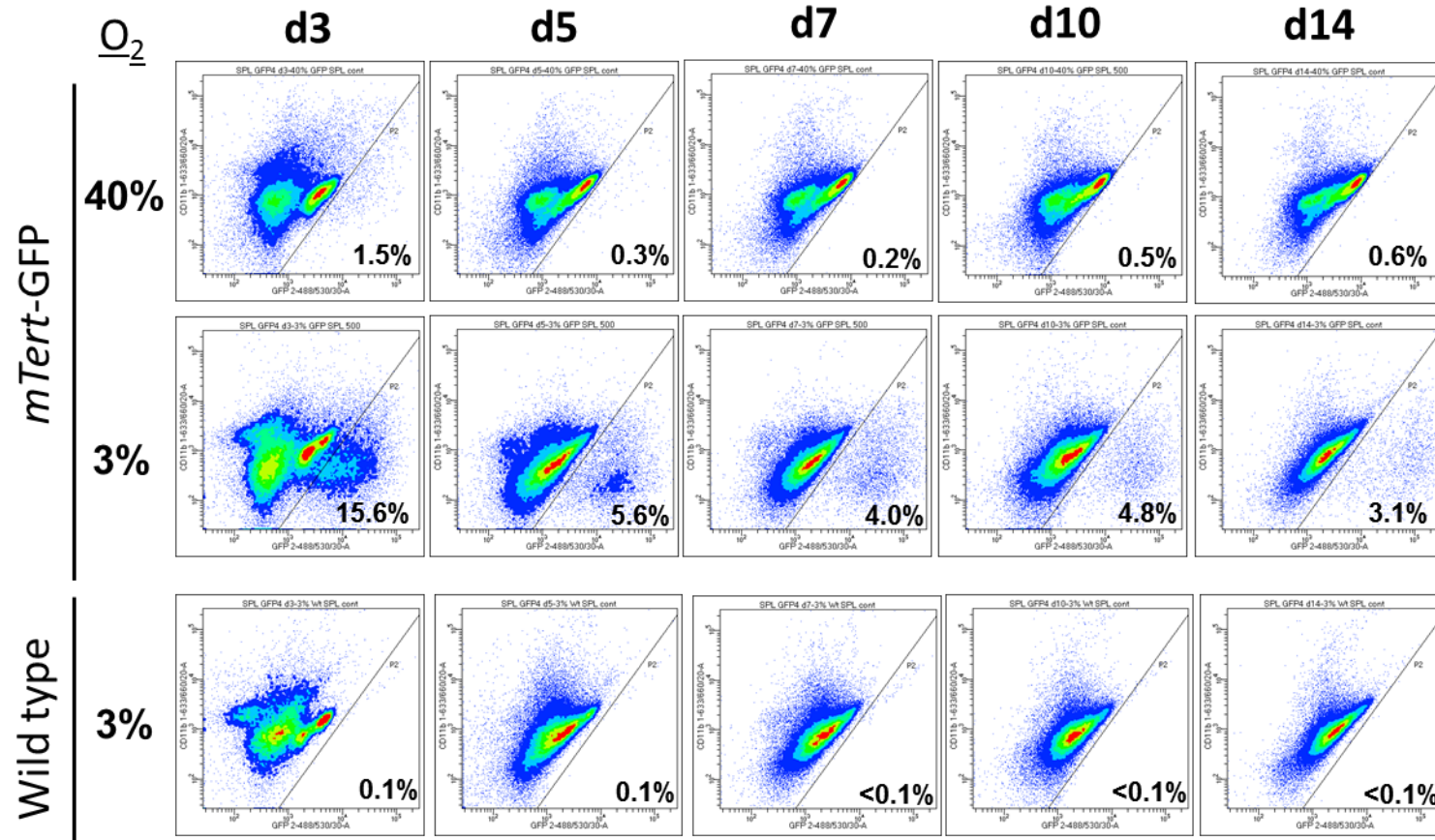
Supplementary Figure VI.



**Supplementary Figure VI. TRAP assay controls.** Positive control: telomerase expressing immortal fibroblast cell line 3T3. For negative control: Splenocytes isolated from the *Tert*<sup>-/-</sup> knockout mouse line.

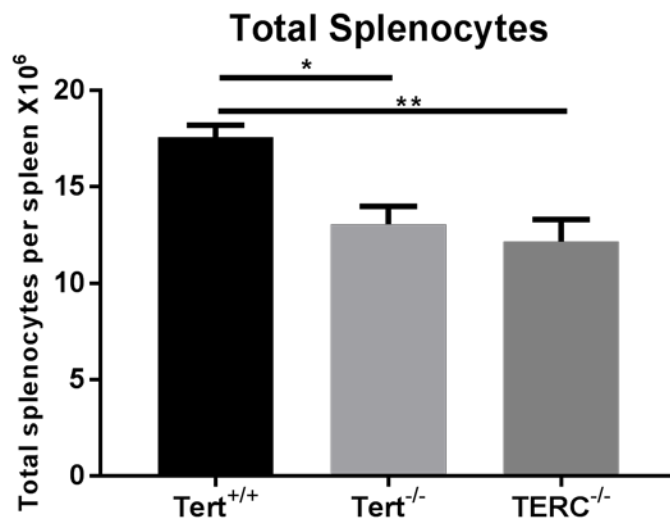


## Supplementary Figure VII



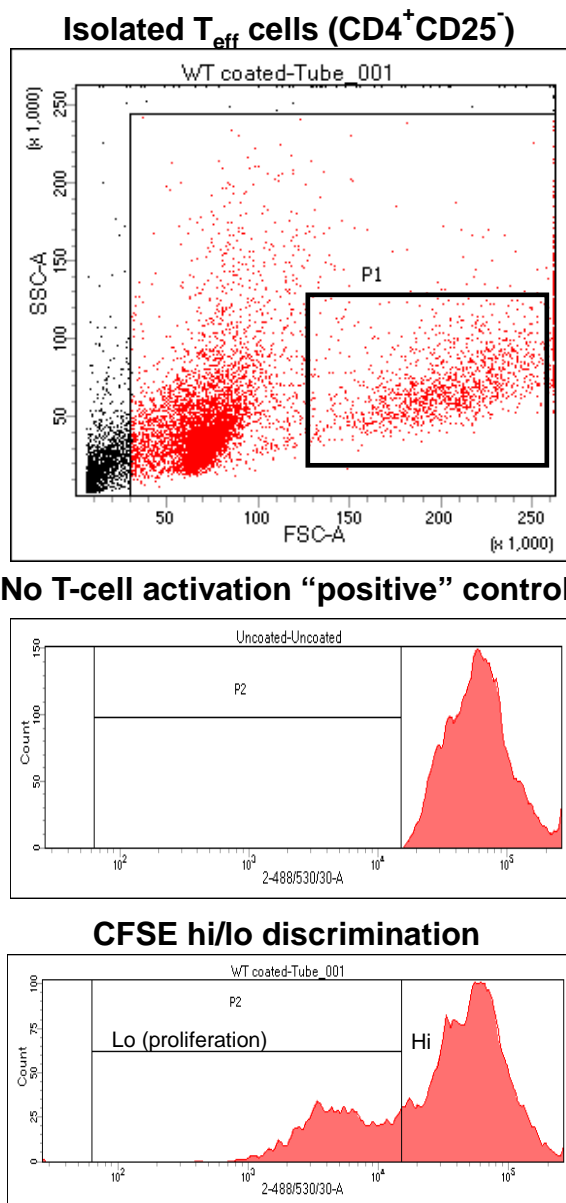
**Supplementary Figure VII. Oxidative stress suppresses telomerase at the level of *mTert* transcription.** Splenocytes were isolated from *mTert*-GFP reporter mice and cultured for 14 days at 3% or 40% and T-cell activation maintained with antibody coated plates. *mTert*-GFP expression was quantified at a single cell level on days 3, 4, 7, 10 and 14 days by flow cytometry. Representative flow dot plots for the percentage of total *mTert*-GFP expressing cells at each time-point and condition as a percentage of total cells. Gating was established using wild type mice as controls with <0.1% positive events in the *mTert*-GFP<sup>+</sup> gate.

**Supplementary Figure VIII.**



**Supplementary Figure VIII. Absolute numbers of Splenocytes obtained during the digestion of each individual spleen.** Following digestion viable cells were counted on a hemocytometer using Trypan blue exclusion to distinguish live cells. N>3 for each experimental condition. \*P<0.05, \*\*P<0.01 using a 1-WAY ANOVA.

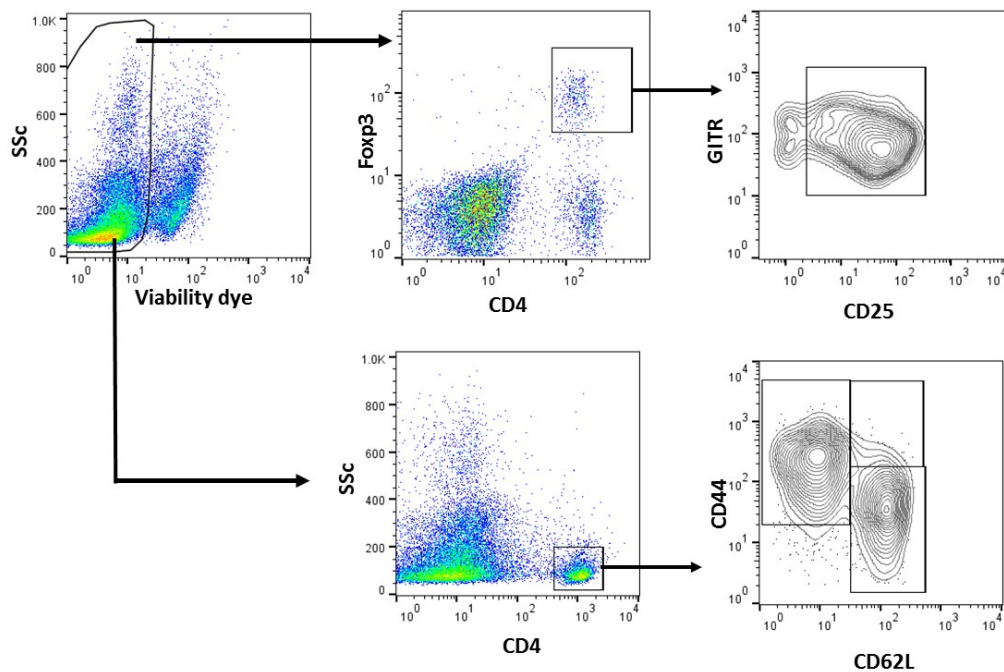
## Supplementary Figure IX



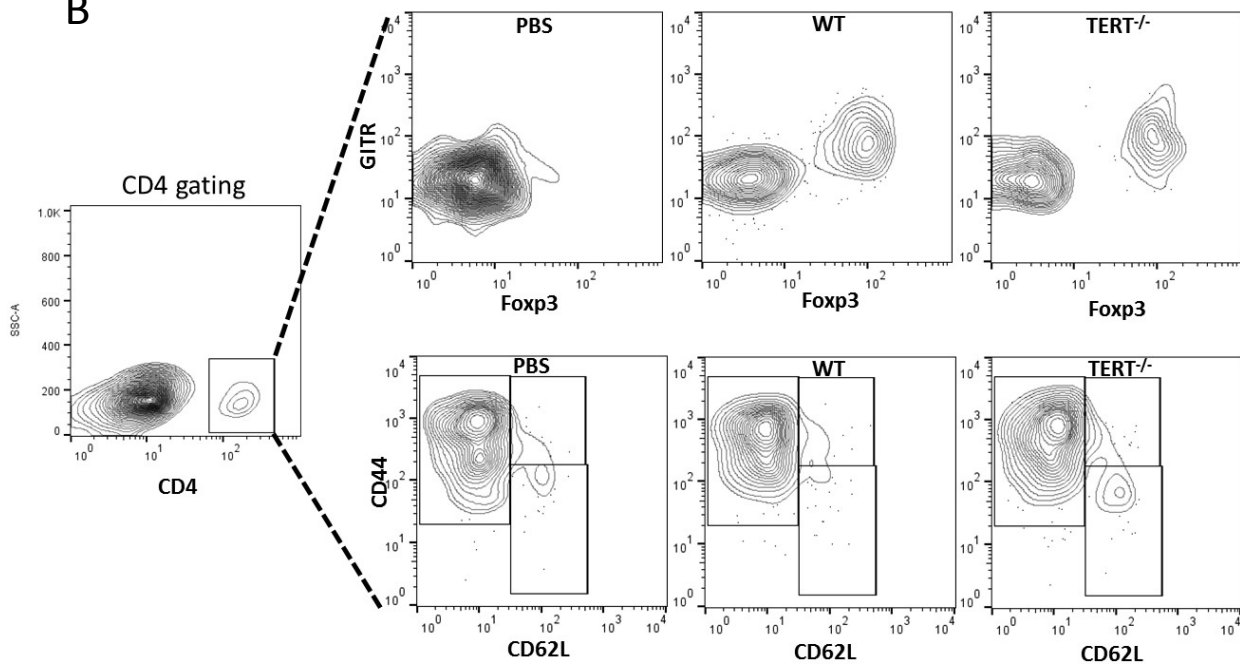
**Supplementary Figure IX. Representative flow plots for CFSE proliferation assay.**

## Supplementary Figure X

A



B



**Supplementary Figure X. Gating strategies A) Identification and quantification of T<sub>reg</sub> (CD4<sup>+</sup> FcγR3<sup>+</sup> CD44<sup>lo</sup>) or T<sub>eff</sub> (CD4<sup>+</sup> CD62L<sup>+</sup> CD44<sup>hi</sup>) in Rag2<sup>-/-</sup> mice.** Rag2<sup>-/-</sup> mice were transplanted with CD28<sup>-/-</sup> splenocytes and either wild type T<sub>reg</sub>, TERT<sup>-/-</sup> T<sub>reg</sub> cells or PBS as control (no transplanted T<sub>regs</sub>). Cells isolated from either spleen or lymph nodes were gated for viability (DAPI). **A)** Viable cells were either i) analysed for FcγR3 vs CD4 antibody labelling or ii) Gated based on CD4 antibody staining followed by subsequent analysis for CD44 and CD62L. **B)** Representative example of flow cytometry plots of T<sub>reg</sub> (CD4<sup>+</sup> FcγR3<sup>+</sup> CD44<sup>lo</sup>) or T<sub>eff</sub> (CD4<sup>+</sup> CD62L<sup>+</sup> CD44<sup>hi</sup>) found in Rag2<sup>-/-</sup> mice transplanted with CD28<sup>-/-</sup> splenocytes and either wild type T<sub>reg</sub>, TERT<sup>-/-</sup> T<sub>reg</sub> cells or PBS control (no transplanted T<sub>regs</sub>).

**Supplementary Table I Details of mouse strain and genetics used in each study.**

<b>Figure</b>	<b>Subfigure</b>	<b>Strain</b>	<b>Genetics</b>
<b>1</b>	A	C57BL/6J	Wild type
	B	C57BL/6J	Wild type
	C	C57BL/6J	Wild type
	D	C57BL/6J	Wild type
	E	C57BL/6J	Wild type
	F	C57BL/6J	Wild type
<b>2</b>	A	B6.129S-Tert, tm1Yjc/J	Wild type littermates <b>F1</b>
	B	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b>
	C	B6.129S-Tert, tm1Yjc/J	Wild type littermates <b>F1</b>
	D	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b>
	E	B6.129S-Tert, tm1Yjc/J	Wild type litter mates <b>F1</b>
	F	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b>
	G	C57BL/6J	Wild type
	F	C57BL/6J	Wild type
<b>3</b>	A	B6.129S	<i>mTert</i> -GFP
	B	B6.129S	<i>mTert</i> -GFP
	C	B6.129S	<i>mTert</i> -GFP
<b>4</b>	A	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
	B	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
	C	B6.129S-Tert, tm1Yjc/J	TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
	D	T <sub>regs</sub> = B6.129S- Tert, tm1Yjc/J	T <sub>regs</sub> = TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
		T <sub>eff</sub> = B6.129S- Tert, tm1Yjc/J	T <sub>eff</sub> = Wildtype =litter mates
	E	T <sub>regs</sub> = B6.129S- Tert, tm1Yjc/J	T <sub>regs</sub> = TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
		T <sub>eff</sub> = B6.129S- Tert, tm1Yjc/J	T <sub>eff</sub> = Wildtype litter mates
	F	C57BL/6J	Wild type



<b>5</b>	A -G	Recipient mice = C57BL/6  CD28 <sup>-/-</sup> Donor mice = C57BL/6J  Tregs = B6.129S	Recipient mice = Rag2 <sup>-/-</sup> /ApoE <sup>-/-</sup>  CD28 <sup>-/-</sup> Donor mice = CD28 knock out  Tregs = TERT <sup>-/-</sup> <b>F1</b> or Wild type litter mates
<b>6</b>	A	B6.Cg-Terc, m1Rdp/J (Blasco et al.,131 1997).	TERC <sup>-/-</sup> <b>F1</b> or Wild type litter mates
	B	B6.Cg-Terc, m1Rdp/J (Blasco et al.,131 1997).	TERC <sup>-/-</sup> <b>F1</b> or Wild type litter mates
	C	B6.Cg-Terc, m1Rdp/J (Blasco et al.,131 1997).	TERC <sup>-/-</sup> <b>F1</b> or Wild type litter mates

## **Detailed Methods**

### **Splenocyte and CD4 cell isolation**

Splenocytes were isolated from mice spleens by Ficoll-hypaque density gradient (using Biochrom AG Biocoll L 6113/5) and prepared at  $2 \times 10^6$ /ml in supplemented RPMI 1640. For CD4<sup>+</sup> cell Isolation, following splenocyte isolation, cells were labelled cells with anti-CD4 (L3T4) labelled magnetic MicroBeads (Miltenyi Biotec 130-049-201) and passed through magnetic column. CD4<sup>+</sup> expressing cells were flushed from the column and collected. To improve CD4<sup>+</sup> T-cells purity, fraction the CD4 positive cell fraction was cells again passed down the column two more times.

### **Splenocyte cultures and growth curves**

Splenocytes were cultured in a 24-well plate ( $2 \times 10^6$  cells/2ml/well). Cultured cells were expanded using (50% volume) complete medium RPMI 1640 with 0.5 mmol/L 2-mercaptoethanol, 25 mmol/L Hepes Buffer, 1.5µg/ml IL-2 (AE5212101, RD,USA)) and 10% FBS. MACSibead mouse T-cell expansion beads (Miltenyi 130-093-627) were added to the remaining medium at a ratio of 1:1 beads per cell (i.e.  $4 \times 10^6$  cells and  $4 \times 10^6$  beads/2ml) as described<sup>1</sup>. 50% of the volume of each sample was transferred to a new well every 2-3 days and 50% fresh medium added to maintain T-cell activation beads. Cells were counted under a microscope using the Neubauer chamber (hemocytometer) and Trypan blue exclusion to identify live cells. Cells were re-activated on a seven-day cycle. When the cell count reached  $10 \times 10^6$  cells, the suspensions were collected, mixed and transferred to a 6-well plate. These were centrifuged to 50% volume and the supernatant aspirated. The cells were then re-suspended in fresh medium. When the cell count reached  $24 \times 10^6$  in the 6-well plate, they were transferred to a 50ml (25cm<sup>2</sup>) culture flask and re-suspended in 50ml complete mouse medium for further cell culture expansion. CD4<sup>+</sup> T-cells were isolated by immunomagnetic bead-selection from total splenocytes. CD4<sup>+</sup> T-cells underwent a similar process. CD4<sup>+</sup> T-cells were first cultured in a 96-well plate ( $2 \times 10^5$  cells/200µl/well) when the cell count reached  $2 \times 10^6$  they were transferred to a 24-well plate ( $2 \times 10^6$ /2ml/well). Following this, the same process was used as for the splenocyte expansion. T cells from either TERT<sup>+/+</sup> or TERT<sup>-/-</sup> mice (n=3) were seeded at  $2 \times 10^5$  cells/well (volume = 200µl/well) in a 96-well plate. TA-65 activator (TA65®) is a telomerase activator purified from *Astragalus membranaceus*<sup>2</sup> and provided by TA-Science Inc., New York, USA). BIBR 1532 (Tocris Bioscience), a telomerase inhibitor,<sup>3</sup> was dissolved in DMSO and used as the indicated concentration.

### **Cell culture for analysis by flow cytometry**

For experiments requiring flow cytometry analysis, wild-type and *Tert*<sup>-/-</sup> mouse splenocytes (n=3 mice in of each genotype) were seeded at  $1 \times 10^6$  cells/well in a 6-well plate coated with anti-CD3 (BD 553238) and anti-CD28 (BD 553295) antibodies and DAPI exclusion to allow gating for viability At the end of experiments splenocytes were stained with four fluorochrome conjugated antibodies; CD45R (PerCP), Cd11b (APC), CD4 (PE-Cy) and CD8 (Pacific Blue) to determine the proportions of each cell type in a sample. In our hands, ≥98% of CD4<sup>+</sup> T cells can be identified as T cells by CD3<sup>+</sup> staining (data not shown).

## Flow cytometry

All flow cytometry was measured on FACSCanto II (BD) and analysed using FACS Diva software.

## DHE and Mitosox staining

Dihydroethidium and Mitosox are established methods to measure superoxide levels.<sup>4, 5</sup> Cells were labelled with 10  $\mu$ M dihydroethidium (Molecular probes) as described<sup>6</sup> or 5  $\mu$ M Mitosox Red (Molecular Probes). Flow cytometry was then used to measure the median fluorescence intensity of 200,000 cells in the appropriate channel compared to unstained controls.

## Quantification of *mTert*-GFP<sup>+</sup> cells

Cells were isolated from spleens of *mTert*-GFP mice ( $n > 5$  per experiment) using complete RF10 (RPMI 1640 with 0.5 mmol/L 2-mercaptoethanol, 25 mmol/L Hepes Buffer, 10% FBS). Lymphocytes were enriched using a Ficoll-Hypaque density gradient.  $1 \times 10^6$  cells were collected and stained using anti-CD4, anti-CD8, anti-CD11b and anti-CD45 antibodies at 0, 3, 5, 7, 10 and 14 days. For culture studies cells isolated and prepared from *mTert*-GFP mouse spleens, as above, and added to plates labelled with anti-CD3 (BD 555338) and anti-CD28 (BD 555727) at a concentration of  $2 \times 10^6$  cells per well. Cells were cultured in complete RF10 with the addition of IL-2 (1.5  $\mu$ g/ml) at either 3% or 40% oxygen. DAPI exclusion was used to allow gating for viability, GFP expression was quantified relative to the total CD4, CD8, CD11b or CD45 cell populations using a FACSCanto II, running FACS Diva software. For each experimental group/time-point 50,000 live events were collected. Negative wild type controls were used to establish gates for flow analysis.

## Quantitative PCR (qPCR)

Total mRNA was isolated from cells and cDNA was transcribed using random primers and the GoScript<sup>™</sup> Reverse Transcriptase kit (Promega). Gene transcripts were quantified using TaqMan qRT-PCR on an Applied Biosystems 7900HT Real-Time PCR system. Assays were run in triplicate and IFN-gamma (Applied Biosystems primer no. MN01168134\_M1, 1340467) and IL2 Applied Biosystems primer no (Mm00434256\_m1 4331182) (transcript expression was quantified using the comparative CT relative to Rm18s (Applied Biosystems primer no Mm03928990\_g1, 1341105) transcript expression. Data were analysed using Step One Software v2.0.

## TRAP-PCR ELISA

Enzyme immunoassay for the semi-quantitative determination of telomerase activity was performed using Telomeric Repeat Amplification Protocol (TRAP) kit (Roche), as per the manufacturer's instructions. 500ng protein lysate was used for each reaction. The Elisa was analysed at 450nm absorbance using an Omega spectrophotometer reader (BMG Labtech).

## Detection of Regulatory T cells

Following splenocyte isolation cells were labelled using the T<sub>reg</sub> Detection Kit (Miltenyi Biotec, Auburn, CA) as per manufactures instructions. Briefly, cells were fixed, permeabilized, and labelled with anti-CD4-FITC (clone: GK1.5), anti- CD25-PE (7D4) and Anti-FoxP3-APC (3G3). DAPI exclusion was used to allow gating for viability. Labelled cells were analysed using a FACS Canto II (BD Biosciences) and FACS diva software (BD Biosciences).

### **Isolation of T cells responder CD4<sup>+</sup>CD25<sup>-</sup> and T<sub>reg</sub> cells CD4<sup>+</sup>CD25<sup>+</sup>**

Mouse CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell populations were isolated from mouse PBMC suspension using a CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> isolation kit, according to the manufacturer's protocols (Miltenyi, UK). Briefly, splenocytes were suspended in PBS containing 0.5% BSA and 2 mmol/L EDTA (pH=7.2) and then incubated with a biotin-antibody cocktail against CD8a (Ly-2; isotype: rat IgG2a), CD11b (Mac-1; isotype: rat IgG2b), CD45R (B220; isotype: rat IgG2a), CD49b (DX5; isotype: rat IgM) and Ter-119 (isotype: rat IgG2b) for 20 min at 4°C. Microbead-conjugated, antibiotin mAb (isotype: mouse IgG1) was added to allow depletion of non-CD4<sup>+</sup> T cells. In parallel, the cells were stained with PE-labeled anti-CD25 mAb. The cell suspension was loaded on a LD column (Miltenyi Biotec), which is placed in the magnetic field of a MACS separator, and then the unlabelled splenocytes were run through. The remaining fraction in the column is the enriched CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD25<sup>-</sup> / CD4<sup>+</sup>CD25<sup>+</sup>). For the isolation of CD4<sup>+</sup>CD25<sup>+</sup> T cells, the PE-labelled CD25<sup>+</sup> T cells in the enriched CD4<sup>+</sup> T cell fraction were labelled magnetically with anti-PE MicroBeads and separated by MACS sorting.

### **Carboxyfluorescein succinimidyl ester (CFSE) proliferation assay**

Purified responder T cells (CD4<sup>+</sup>CD25<sup>-</sup>) were re-suspended in PBS (0.1% BSA) at  $2 \times 10^6$  cells/ml and incubated with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) (final concentration: 1µmol/L) for 7 min at 37°C. Cells were washed in cold PBS and re-suspended in culture medium for 15 min to stabilize the CFSE staining. CFSE staining was analyzed using a FACS Canto II (BD Biosciences) and FACS diva software (BD Biosciences).

### **T<sub>reg</sub> Suppression assay**

Murine splenic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were isolated from WT, TERT<sup>-/-</sup> and TERC<sup>-/-</sup> mice using a MACS sorting isolation kit as described above. The CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated and stained with CFSE to be used as responder T cells.  $1 \times 10^5$  responder cells (CD4<sup>+</sup>CD25<sup>-</sup>) were added per well in RPMI-1640 medium supplemented with 10% FCS and 1.5µg/ml IL-2 (R&D 202-IL) in 96-well, round-bottomed plate which had been pre coated with anti CD3 (BD 553238) and CD28 (BD 553295) at 1µl/ml PBS at 4°C. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were subsequently added to each well at the indicated ratio. Cells were co-cultured in complete medium at 37°C and 5% CO<sub>2</sub> for 72 h. After incubation cells were harvested and the proliferation of CD4<sup>+</sup> responder T-cells was measured on FACS Canto flow cytometer (BD Biosciences) and analysed with FACS diva software (BD Biosciences).

### **Atherosclerosis experiments**

Rag2<sup>-/-</sup> ApoE<sup>-/-</sup> mice were transplanted with 10<sup>7</sup> splenocytes from CD28<sup>-/-</sup> mice and either PBS, or 10<sup>6</sup> CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (isolated by magnetic separation as above) from either Tert<sup>-/-</sup> mice or wild type littermates. Mice were fed an atherogenic Western diet (21% fat, 0.15% cholesterol) for 7 weeks. Atherosclerosis was quantified in the aortic root as described previously<sup>7</sup>. Briefly, ten serial cryosections spanning the root were stained with Oil Red O. Microscope images were obtained and Oil Red O staining quantified using a Leica microscope and LAS AF software. Plasma cholesterol levels were measured using a commercial cholesterol kit (Biomerieux).

## Statistical Analysis

Following a test for normality statistical analysis was performed as appropriate and indicated in the legend of each figure, data are presented as mean ± SEM or as dot for individual experiments with a line representing the median. A Mann-Whitney U-Test was used to compare groups of two and two-way ANOVA with Bonferroni post-hoc analysis was used to compare groups of three or more. Statistical significance was set at p<0.05.

## References

1. Bennaceur K, Atwill M, Al Zhrany N, Hoffmann J, Keavney B, Breault D, Richardson G, von Zglinicki T, Saretzki G, Spyridopoulos I. Atorvastatin induces t cell proliferation by a telomerase reverse transcriptase (tert) mediated mechanism. *Atherosclerosis*. 2014;236:312-320
2. Bernardes de Jesus B, Schneeberger K, Vera E, Tejera A, Harley CB, Blasco MA. The telomerase activator ta-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence. *Aging Cell*. 2011;10:604-621
3. Beyer AM, Freed JK, Durand MJ, et al. Critical role for telomerase in the mechanism of flow-mediated dilation in the human microcirculation. *Circ Res*. 2016;118:856-866
4. Owusu-Ansah E, Yavari A, Banerjee U. A protocol for \_in vivo\_ detection of reactive oxygen species. 2008
5. Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, Pacher P. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nat Protoc*. 2007;2:2295-2301
6. Ashor AW, Chowdhury S, Oggioni C, Qadir O, Brandt K, Ishaq A, Mathers JC, Saretzki G, Siervo M. Inorganic nitrate supplementation in young and old obese adults does not affect acute glucose and insulin responses but lowers oxidative stress. *J Nutr*. 2016;146:2224-2232
7. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res*. 2001;89:E41-45